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AMANTADINE AS AN ANTIMALARIAL

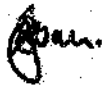
by SANDRA GAIL EVANS

Thesis submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, in fulfilment of the requirement for the degree of Doctor of Philosophy

May 1996

DECLARATION BY CANDIDATE

I declare that this report is my own work and that it has not been submitted for any degree to any other university.



S G Evans

May 1996

DEDICATION

To my father, Raymond Allan Evans, who opened the door to the world of science through his love of books and the outdoors.

ABSTRACT

Amantadine is used clinically to treat Influenza A viral infections and Parkinsons's disease. The lysosomotropic nature of amantadine suggested potential as an antimalarial. The aim of this study was to determine and characterise the antimalarial activity of amantadine. Sensitivity tests were carried out *in vitro* against different *Plasmodium falciparum* strains. The antimalarial activity was further investigated using a variety of techniques. Amantadine was found to effectively inhibit intraerythrocytic growth of a chloroquine-resistant strain of *P.falciparum* at clinically attainable concentrations. The sensitivity to amantadine was found to be inversely correlated with chloroquine sensitivity. This relationship differs from that seen with resistance "reversal" agents and thus amantadine was classified in a new category of antimalarial drugs termed "inverse resistance" drugs. Amantadine does not interfere with the activity of standard antimalarial compounds and may possible enhance their activity. All stages of the parasite are susceptible to amantadine, however, only exposure to the late stages results in irreversible damage. Amantadine accumulates in the food vacuole producing morphological changes typical of a lysosomotropic agent, however, amantadine does not interfere with functioning of the food vacuole. Sensitivity to amantadine is associated with a membrane component. It does not block parasite induced pores directly but is able to interfere with nutrient uptake either by a direct membrane effect or indirectly by inhibiting secretion of parasite proteins into the erythrocyte membrane. Amantadine does not block protein synthesis. The antimalarial properties of amantadine are unique and complex. A membrane interaction is implicated in the mechanism of action of amantadine.

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- God, who makes all things possible

ABBREVIATIONS

AMA	Amantadine
ANS	8-anilinonapthalene-1-sulfonic acid
BSA	Bovine serum albumin
°C	Degree celsius
CHLOROQUINE	Chloroquine
DMSO	Dimethylsulphur oxide
dpm	Disintegrations per minute
g	Grams
HA	Haemagluttinin
HEPES	4-(2-hydroxyethyl)-1-peperaxinethane sulphoric acid
HF	Halofantrine
IC_{50}	Concentration resulting in 50% inhibition of parasite growth *in vitro*
min	Minutes
MQ	Mefloquine
NH_4Cl	Ammonium chloride
PBS	Phosphate buffered saline
PBS-G	Phosphate buffered saline supplemented with 10 mM glucose
PQ	Primaquine
pRBC	Parasitized red blood cell

PYR	Pyrimethamine
QN	Quinine
r	Regression coefficient
RBC	Red blood cell
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
TRIS	Hydroxymethylamdionmethane
µl	Microlitre
VER	Verapamil

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LIST OF PUBLICATIONS

PRESENTED AT CONFERENCES AND PUBLISHED IN CONFERENCE PROCEEDINGS

Evans SG, Havlik I. Effect of amantadine, an antiviral, on *Plasmodium falciparum* growth *in vitro*. **South African Pharmacology Society 26th Annual Congress 1992**

Endeacott W, Evans SG, Havlik I. The combined effect of amantadine and three standard antimalarials - chloroquine, quinine and pyrimethamine on the growth of *Plasmodium falciparum in vitro*. **South African Pharmacology Society 26th Annual Congress 1992**

Evans SG, Havlik I. The influence of medium pH on antimalarial activity of amantadine. **Parasitological Society of Southern Africa 22nd Annual Congress 1993**

Evans SG, Havlik I. The effect of MDP on the clinical course and outcome of *Plasmodium berghei* infection in mice. **South African Pharmacology Society 27th Annual Congress 1993**

Blanchard CL, Van Zyl RL, Evans SG, Havlik I. Analysis of heme-proteins of *Plasmodium falciparum* using SDS polyacrylamide gel electrophoresis. **South African Pharmacology Society 27th Annual Congress 1993**

Evans SG, Havlik I. *Plasmodium falciparum* : Inhibition of *in* vitro growth by amantadine **Parasitological Society of Southern Africa 23rd Annual Congress 1994**

Evans SG, Havlik I. Identification of the stages of *Plasmodium falciparum* sensitive to amantadine. **XIIth International Congress of Pharmacology, Montreal 1994**

Evans, SG, Havlik. Alkalinization of the food vacuole of malaria parasites by amantadine is not correlated with its antimalarial activity. **South African Pharmacology Society 28th Annual Congress 1994**

Brown C, Rovelli S, Havlik I, Evans SG, Kaneko Y. The effect of curdlan sulphate on *Plasmodium berghei* infection in Balb/c mice. **South African Pharmacology Society 28th Annual Congress 1994**

Evans SG, Havlik I. The effect of amantadine on permeation pathways induced in the host cell membrane by the malaria parasite. **South African Pharmacology Society 29th Annual Congress 1995**

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Evans SG, Havlik I. *Plasmodium falciparum* : effects of amantadine, an antiviral, on chloroquine-resistant and -sensitive parasites *in vitro*, and its influence on chloroquine activity. **Biochemical Pharmacology** (1993) 45(5):1168-1170

Evans SG, Havlik I. *Plasmodium falciparum* : *in vitro* drug interactions between amantadine and classic antimalarials. **Transactions of the Royal Society of Tropical Medicine and Hygiene** (1994) 88:683-686

Evans SG, Havlik I. Extracellular pH modulates *in vitro* potency of amantadine against *P.falciparum*. **Am J Trop Med Hyg** (1996) 54(3):232-236

PREFACE

Malaria, a parasitic disease caused by protozoa of the genus *Plasmodium*, counts among the worst scourges of mankind. The global incidence of malaria is estimated to be 110 million persons per year, some 270 million carry the parasite and the number of people at risk is 2100 million. The number of deaths in sub-Saharan Africa alone is estimated at 1.5-3 million annually.

A world-wide malaria eradication programme was begun in the 1950s, by the 1970s significant progress had been made, with malaria having been eliminated from Europe, part of Asia and large areas of South America. However, the emergence of drug resistant malaria parasites, the failures of vector control due to insecticide resistance and political unrest have resulted in a resurgence of malaria in the 1980s. The WHO estimates the number of infected people is rising at a rate of 5 % per annum.

Malaria imposes an economic burden on households. Scarce resources must be used to ameliorate the consequence of infection, both as direct costs - for example, for diagnosis and treatment - and as indirect costs in the form of morbidity and mortality that can reduce the time available for productive pursuits and the productivity of the times so allocated. In addition there is growing evidence that the impact can be more subtle - retarding physical growth, development of cognitive skills and educational performance and participation. This reduces the longer term economic potential of

individuals and perhaps society. By restricting economic potential malaria can exacerbate existing inequalities in society to a much greater extent than non-communicable diseases.

The problem is mainly a Developing World problem. One major difficulty is the attitude of the pharmaceutical industry, which has been beating a steady retreat from the development of new anti-parasitic products. As the cost of bringing a drug onto the market have spiralled upwards, companies have become less willing to invest in diseases of poverty, where there is little prospect of large profits. Current strategies to combat malaria include the screening of drugs that have been developed for other purposes for anti-parasitic activity.

The purpose of this thesis is to examine and define the antimalarial activity of amantadine; a drug developed in the 1960s for use against influenza A viral infections. The thesis is divided into a number of different parts. Chapter one is an extensive literature review, dealing with all aspects of the drug amantadine hydrochloride. The second chapter looks at the methodology used to obtain parasites for experimental purposes. Although similar methods were used throughout this study to obtain parasite samples, sample processing varied between chapters so protocols for each experimental chapter are detailed in the method sections within that chapter. The first experimental chapter defines the antimalarial activity of amantadine and other

antimalarial agents against departmental parasite strains. The second chapter, investigates the potential of amantadine therapeutically, by analysing the effect of amantadine in combination with other antimalarials. The third experimental chapter records the morphological changes associated with exposure of the parasite to amantadine. The remaining experimental chapters seek to define the mechanisms involved in the action of amantadine.

Parts of this thesis have been presented at a number of conferences, both local and international. Sections of this thesis have also been published in full, while others have been submitted and still others are in preparation.

CHAPTER ONE - AMINOADAMANTANES

1.1 CHEMISTRY

Aminoadamantanes are lipophilic quasi-spherical compounds. The geometric relationship between the polar portion and the cage-like apolar portion of amantadine is unusual as compared with other drugs (figure 1.1) Amantadine hydrochloride is a stable crystalline, water soluble amine salt with a pK_a of 10.1. Four aminoadamantanes are currently in clinical use: amantadine (1-adamantanamine), rimantadine (α-methyl-1-adamantanethylamine), memantine (1-amino-3,5-dimethyladamantane) (Parkes, 1974) and tromantadine (Diezel *et al.*, 1993).

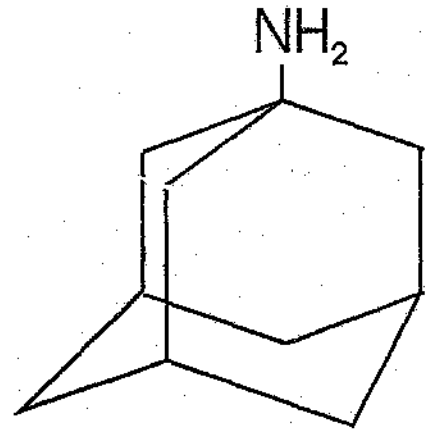


Figure 1.1: Structure of amantadine

Amantadine was first synthesised more than 30 years ago and initially introduced as an antiviral agent (Davies *et al.*, 1964). Clinical observations indicated aminoadamantanes ameliorated symptoms of Parkinson's disease (Schwab *et al.*, 1969, Parkes *et al.*,

1970). The suggested list of clinical applications for the aminoadamantanes has grown in the ensuing years to include various other cerebral disorders such as neuroleptic malignant syndrome, spasicity and dementia.

1.2 BASIC PHARMACOLOGY

1.2.1 ENTRY AND INTRACELLULAR ACCUMULATION

Amantadine causes vacuolation in cells and a gross expansion of the intracellular vesicular system due to its weak base character. Briefly, amantadine penetrates cells in the uncharged form and rapidly loads the cytosol to a concentration in the order of the extracellular concentration. Then amantadine accumulates within the acidic organelles (notably the lysosome) to much higher concentrations because it becomes trapped in the hardly permeable form (Richman *et al.*, 1981). The term "lysosomotropic" designates all substances taken up selectively into lysosomes, irrespective of their chemical nature or mechanism of uptake (De Duve *et al.*, 1974).

1.2.2 DIRECT EFFECT OF AMANTADINE ON ENZYMES

1.2.2.a Phospholipases

Amantadine induces phospholipidosis in cells *in vitro* and *in vivo* (Burmester *et al.*, 1987). Drug-induced lipidosis is due to impaired lysosomal lipid degradation. Amantadine inhibits catabolism by forming a complex with phospholipids: this complex resists hydrolysis (Hostetler and Matsuzawa, 1981).

1.2.2.b P-450 cytochrome

Amantadine, at high doses, exerts depressive effects on the drug metabolising enzyme activities. An *in vivo* mechanism that results in lowered levels of microsomal cytochromes P-450 and b_5 either by decreasing the synthesis of these proteins or by accelerating their degradation is involved (Belanger *et al.*, 1979).

1.2.3 EFFECT OF AMANTADINE ON MEMBRANE STRUCTURE AND FUNCTION

1.2.3.a Drug-lipid interactions

Amantadine has a low affinity towards phospholipids as determined by the calcium displacement method (Burmester *et al.*, 1987). Although, amantadine is able to produce a downward shift of the transition temperature, (the temperature at which a membrane "melts" to adopt a fluid crystalline state - the temperature at which this happens is dependent on the packing of hydrocarbon tails and related to the ratio of drug:lipid) the effect is small (ΔT_t of 3°C) (Burmester *et al.*, 1987). The above evidence infers that amantadine does not have strong drug-lipid interactions, probably due to the geometry of amantadine, which hinders an effective intercalation of the drug between the phospholipids. X-ray diffraction and neutron profile studies orientates amantadine in the bilayer. The hydrophobic cyclic region of the drug is located between the phosphate and ester linkages of the phospholipids and the NH_3^+ group protrudes into the water space. The cyclic carbon component is orientated on the

hydrophobic side of the phospholipid headgroup allowing for interaction with the headgroup. The effects of amantadine on bilayer structure and stability are likely to be complex and to be dependent upon intrinsic and environmental factors (Duff *et al.*, 1993).

1.2.3.b Ion channels

Amantadine interferes with many membrane structures, preferentially with ion channels. The channels blocked by amantadine include the voltage-dependent Na^+ channel (Netzer *et al.*, 1991), glycine-operated Cl^- channel (Lampe and Bigalke, 1991), the nicotinic acetylcholine ion channel (Masuo *et al.*, 1986), ATP-regulated K^+ channel (Ashcroft *et al.*, 1991), the Ca^{2+}-permeable channel activated by 5-HT (Reiser and Koch, 1989), the NMDA channel (Kornhuber *et al.*, 1991) and a viral proton channel (Pinto *et al.*, 1992).

1.3 PHARMACOKINETICS

1.3.1 ABSORPTION

The oral route is mainly used for amantadine administration. Amantadine is well absorbed from the GI tract. In healthy volunteer's absorption is relatively complete, varying from 86 to 90 % in young healthy adults over a wide range of doses. Peak plasma concentration is directly related to the dose ingested. The time to peak plasma concentration is 1 to 4 hours in healthy young adults. The rate of absorption and bioavailability decreases in healthy elderly adults (Greenblatt *et al.*, 1977).

1.3.2 DISTRIBUTION

Amantadine in plasma is approximately 67% protein bound and the percentage of drug bound is independent of amantadine concentration. The volume of distribution correlates inversely with the dosage of amantadine. The V_d for amantadine exceeds the body volume by a considerable amount inferring extensive tissue binding. Red blood cells sequester substantial amounts of amantadine, with a erythrocyte:plasma ratio of 2.66 ± 0.49 in healthy males. Very little data is available in humans concerning the extravascular distribution of amantadine. Amantadine sequesters in all tissues based on its kinetic distribution space (Greenblatt *et al.*, 1977).

1.3.3 ELIMINATION

The metabolism is limited, consequently elimination is via renal glomerular filtration and active tubular secretion. The elimination half life is between 9-37 hours, with an average of 24 hours. The half life is prolonged in patients with impaired renal function (Greenblatt *et al.*, 1977).

1.4 ANTIVIRAL ACTIVITY OF AMINOADAMANTANES

1.4.1 SPECTRUM OF ACTIVITY

All strains of influenza A virus are sensitive to amantadine. Group B influenza viruses and other respiratory pathogens such as rhinoviruses and respiratory syncytial and adenoviruses are resistant to amantadine. Other viruses including parainfluenza,

pseudorabies, rubella, murine and avian tumour viruses, arena viruses, lymphocytic chorimeningitis, junin virus, dengue and HIV-1 are inhibited by amantadine in tissue culture or experimental animals (Chang and Snydman, 1979). Clinical use of amantadine and rimantadine is limited to use against influenza A viruses. Tromantadine is used clinically as a topical cream against herpes simplex (Ickes *et al.*, 1990, Diezel *et al.*,1993).

1.4.2 INFLUENZA A VIRUS

Amantadine and rimantadine are used for prophylactic purposes in high risk groups during influenza A epidemics (Oates *et al.*, 1990). For all influenza virus strains, the amantadine block to virus replication occurs at the early stage between the steps of virus penetration and uncoating. In addition to the early effect of amantadine, the drug has a second late effect on some subtypes, defined as having a intracellularly cleaved haemagluttinin and a high pH optimum of fusion, for example fowl plague virus. Characterisation of amantadine resistant mutants identified the M_2 protein as the target of their action (Hay *et al.*, 1979).

1.4.2.a M_2 protein

The M_2 protein is a small, 97 amino acid integral membrane protein orientated in membranes such that it has a 24 N-terminal extracellular residues, a 19 residue transmembrane domain and a 54 residue cytoplasmic tail. Single amino acid substitutions at positions 26, 27, 30 and 31 within the transmembrane domain of this

integral membrane component confer drug resistance (Wang *et al.*, 1993). M_2 molecules expressed in *Xenopus* oocytes form amantadine-sensitive channels for monovalent cations in the plasma membrane . Despite its small size and simplicity, the ion channel is regulated by voltage, pH and drugs like amantadine (Tosteson *et al.*, 1994). Amantadine probably acts as an allosteric blocker - binding to part of the channel protein that is not in the pore-forming region. The calculated IC_{50} of amantadine is 0.3 μM, i.e. one amantadine molecule blocks each functional M_2 ion channel and the block has a very slow onset (Wang *et al.*, 1993). M_2-like channels have not been identified in mammalian systems, nor does the M_2 sequence share significant homology to known mammalian proteins (Pinto *et al.*, 1992).

1.4.2.b Role of M_2 in virus uncoating

Figure 1.2 illustrates the process of viral uncoating. (Simons *et al.*, 1982). Briefly, viruses enter cells by endocytosis. The first stage of entry is the attachment of spike glycoproteins to the cell surface. The virus then moves into a "coated pit". The coated pit folds inwards and the cell membrane pinches off around the virus particle to form a vesicle. As the coated vesicle moves into the cytoplasm it loses its clathrin and fuses with an endosome, a large vacuole with a smooth outer surface. The M_2 protein is left behind in the endosomal membrane after fusion. Once the virion particle has been endocytosed, the ion channel activity of the virion-associated M_2 protein permits the flow of ions from the endosome into the virion interior to disrupt the protein-protein

interactions and frees ribonucleocapsid and M_1 protein, priming the virion for dissociation upon penetration into the cytosol (fig 1.3).

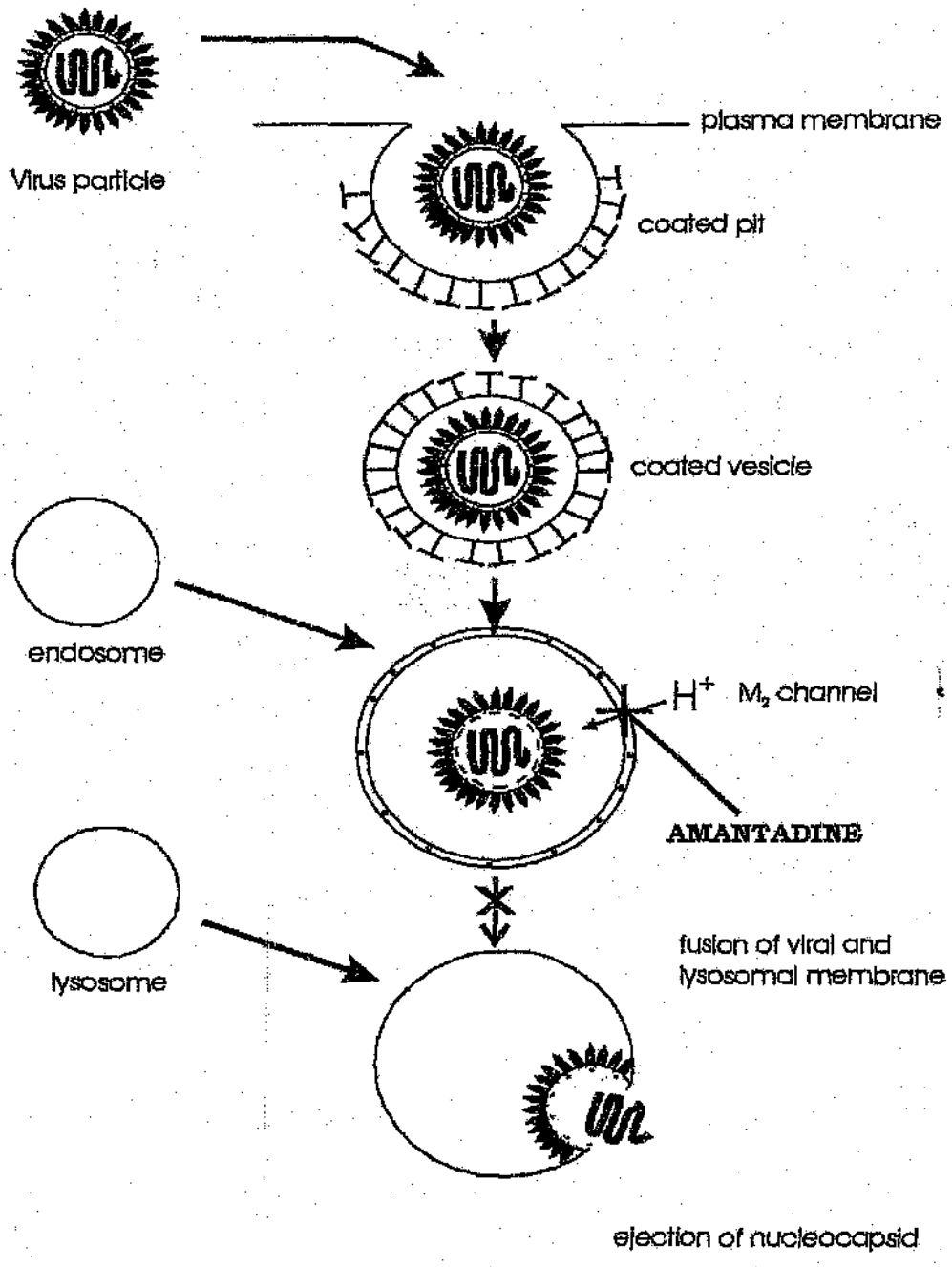


Figure 1.2: Schematic diagram showing amantadine block on virus uncoating

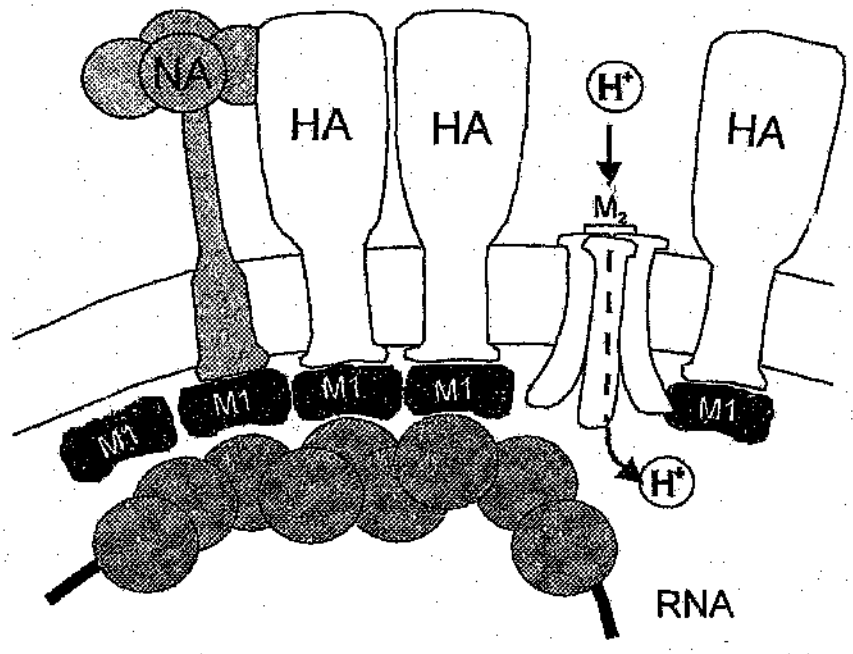


Figure 1.3: Molecular interaction between structual components of influenza virus. HA : hemaglutinin, NA : neuraminidase

The endosome itself then fuses with a lysosome, the degradative vacuole that contains a variety of digestive enzymes at a low pH. The acidity of the interior of the lysosomes induces changes in the viral membrane protein, heamgalutinin, that enables fusion with the lysosomal membrane. The process takes place so quickly that the nucleocapsid is expelled into the cytoplasm without being destroyed by the degradative enzymes of the lysosome. Thereafter, the vRNPs (viral ribonucleic particles) rapidly enter the nucleus and initiate replication and transcription. The release of the nucleocapsid from the lysosome completes the first phase of infection. Amantadine blocks the channel preventing the dissociation of the M_1 protein from the ribonucleoproteins and subsequent viral replication.

1.4.2.c Role of M_2 in virus maturation

Virus maturation comprises replication of the viral RNA, the manufacture and modification of viral proteins and the assembly of the nucleocapsid; figure 1.4 illustrates this process (Simons *et al.*, 1982).

Two different kinds of RNA molecules are manufactured in large amounts; one is a copy of the complete viral nucleic acid molecule, the other contains the genetic information required to synthesise the viral proteins. The membrane proteins are not simply released from the ribosome into the cytoplasm, but are inserted into the membrane of the endoplasmic reticulum, a network of inter connected membranes that plays a critical role in the synthesis and modification of host cell and secretory proteins. The viral protein complex on the endoplasmic reticulum is then transported to the surface of the cell along the same route followed by host cell secretory and membrane proteins i.e. from the endoplasmic reticulum they move to the Golgi apparatus, a complex organelle with several interior spaces enclosed by membranes. In the Golgi apparatus the carbohydrate chains are modified. At this stage the M_2 molecules, embedded in the Golgi membrane, appear to be responsible for elevating the otherwise mildly acidic pH in the trans-Golgi network, by forming proton channels through which the proton gradient between the lumen of these organelles and the cytosol dissipates. The resulting elevation in the luminal pH, facilitates the passage of HA without acid conversion. Amantadine blocks the M_2 channels, consequently the

HA molecules are exposed to low pH and undergo a premature conformational conversion that results in the formation of non-infective virus particle. After the proteins have passed through the Golgi apparatus they are carried to the surface of the host cell and inserted in the cell membrane.

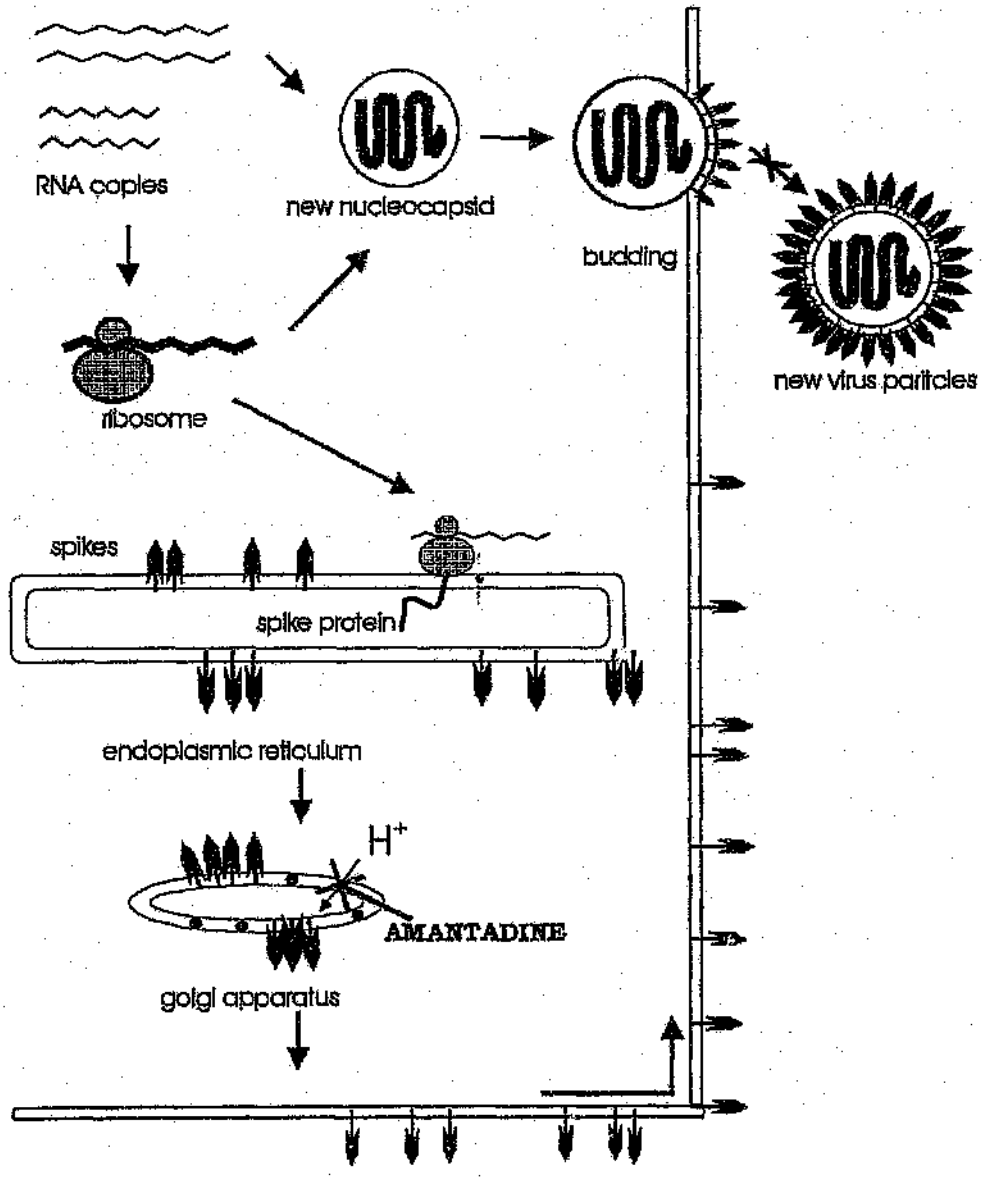


Figure 1.4 : Schematic diagram of virus maturation and block by amantadine

The final phase, the transit of the virus through the animal cell follows the assembly of the nucleocapsids in the cytoplasm of the cell. A section of the membrane, with viral membrane proteins embedded, wraps around the nucleocapsid and the bud releases from the cell. The complete virus particles are released into the space between the cells, from which they move outward toward other cells and spread the infection.

1.5 CEREBRAL DISORDERS

Aminoadamantanes stimulates the *in vivo* release of dopamine. However, the concentration of amantadine needed to demonstrate a significant effect is in the high micromolar range or low millimolar range. Plasma levels of effective doses of amantadine are in the low micromolar range. The clinical effects could not be attributed solely to increased dopamine release. Today, there is considerable evidence that aminoadamantanes act as non-competitive antagonists at the N-methyl-D-aspartate (NMDA) receptor (Bormann, 1989, Kornhuber *et al.*, 1994), and that NMDA receptor antagonism mediates the cerebral clinical effects.

NMDA receptor is an integral membrane protein, it includes recognition domains for glutamate and several endogenous co-agonist and modulatory substances including glycine and polyamines. The receptor also contains a cation-selective pore that serves as a pathway across the neuronal membrane for Na^+, K^+ and Ca^{2+} ions - see figure 1.5. Activation of NMDA receptors by neurotransmitter glutamate is associated with a conformational change in the receptor pore that permits cations to cross the

membrane under the force of their respective electrochemical gradients, resulting in membrane depolarisation and neuronal excitation (Rogawski, 1992). Aminoadamantanes antagonise NMDA responses by an 'open channel' mechanism in which they bind to the ionphore of the NMDA receptor only when it is in the ligand gated open state . Occupancy of the ionphore binding site prev nts cation flux through the channel, thus producing a functional block of NMDA receptor responses. The kinetics of the channel block shows uncompetitive antagonism with fast blocking and unblocking rates, facilitating blockage of enhanced glutamergic activity at the NMDA receptor while leaving physiological transmission of glutamate largely unaffected and thus preventing the psychotic symptoms associated with other NMDA antagonists (Chen *et al.*, 1992).

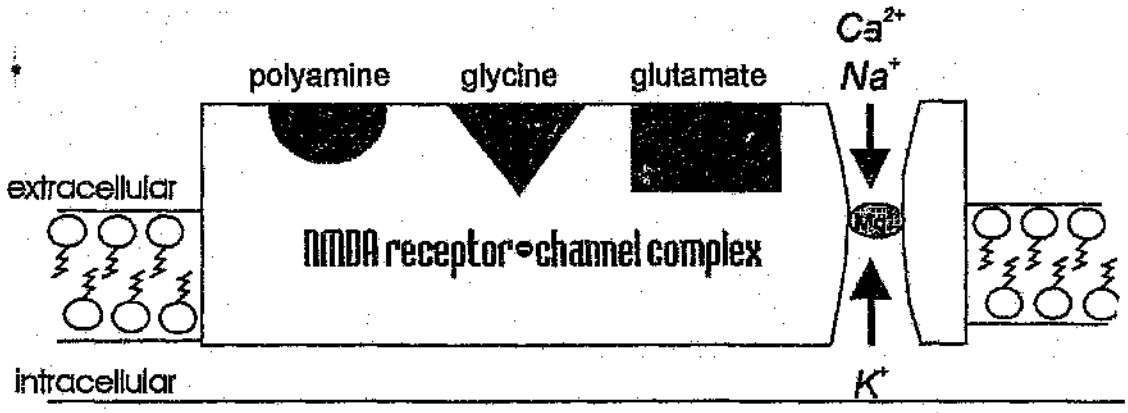


Figure 1.5 : Schematic representation of the NMDA receptor-channel complex

1.5.1 PARKINSONS'S DISEASE

A disturbance of neostriatal dopaminergic function, through loss of dopaminergic inervation, plays an important role in the pathophysiology of Parkinson's disease.

Under physiological conditions, the release of acetylcholine in this brain region is under the inhibitory control of the nigrostriatal dopaminergic input, whereas a corticostriatal, glutamatergic input stimulates acetylcholine release via NMDA receptors. In the case of dopamine deficiency in the striatum, the cholinergic cells become 'disinhibited' and the normal situation can be restored by applying either dopaminomimetic or anticholinergic drugs. NMDA receptors activate the cholinergic cells in the neostriatum - NMDA antagonism would be expected to decrease the output of acetylcholine and thereby display anti-Parkinson's activity (Klockgether and Turski, 1989).

The anti-Parkinson effect is mediated both via NMDA receptor antagonism as well as by a post-synaptic effect. Chronic administration of amantadine enhances D_2 receptor expression in the striatum without changing the affinity of the receptor (Gianutsos *et al.*, 1985). Rojas *et al.*, 1992 described a partially protective effect of amantadine on the MPTP model of Parkinson's disease, suggesting that aminoadamantanes may not only ameliorate the motor manifestations of reduced nigrostriatal transmission but also attenuate disease progression. There is no evidence that the molecular mechanism of MPP^+ toxicity involves NMDA receptors, the molecular target is believed to be complex I of the mitochondrial respiratory chain. However, neurones show an increased susceptibility to NMDA receptor mediated glutamate toxicity during low energy crisis. Inhibition of mitochondrial functioning produces a disturbance in the

intracellular energy level, interfering with the neuron's ability to maintain a resting potential and consequently the Mg^{2+} block of the NMDA receptor (Novelli *et al.*, 1988).

1.5.2 NEUROLEPTIC MALIGNANT SYNDROME

Dopamine receptor antagonists, i.e. neuroleptic drugs are the principle class of drugs that are used in the pharmacology of schizophrenia. The most common side effects of these drugs are symptoms of the extrapyramidal motor system including rigidity, tremor and akinesia termed neuroleptic malignant syndrome (NMS). Amantadine has been used to successfully treat NMS patients (McCarron *et al.*,1982, Amdurski *et al.*, 1983, Gangadhar *et al.*, 1984, Woo *et al.*, 1986).

1.5.3 DEMENTIA

Treatment of dementia concentrates on mobilisation of the cerebral reserves still present in the clinical condition while preventing further progress of the disease. The aim of therapy is to maintain as long as possible an independent life style in the environment to which the patient is accustomed.

1.5.3.a Senile dementia

The pathophysiological process in senile dementia is believed to be due to a loss of glutamergic neurons in the cortex. Memantine brings about a statistically significant and clinically relevant influence on brain function disturbances and improvement in performance deficits in the dementia syndrome patient that particularly improve

independent handling difficult situations in everyday life and social integration. The efficacy appears after only 14 days of treatment and there is a high responder rate (Ditzler, 1991).

1.5.3.b AIDS dementia complex

As many as 2/3 of AIDS patients develop signs of human immunodeficiency virus (HIV) associated cognitive and/or motor complex of which a severe form is AIDS dementia complex (ADC). The pathophysiologic process that produces ADC is unclear. It is believed to be due in part to neuronal injury caused by the viral protein gp120, or fragments thereof, shed from HIV-1. Although gp120 has no direct effect on the NMDA receptor it sensitises cells to NMDA mediated toxicity. Memantine completely prevents injury *in vitro*, suggesting it has therapeutic potential (Lipton, 1992).

1.5.4 SPASICITY

Memantine can prevent seizures and neurotoxic cell death induced by various chemicals and electrical means and potentiates the anticonvulsant actions of other anti-epileptic drugs (Apland and Cann, 1995).

1.5.5 OTHER

1.5.5.a Fatigue

Limited studies suggest that amantadine is effective in relieving fatigue associated with multiple sclerosis (Kemp and Gora, 1993) and post-polio syndrome (Dawson, 1991). The basis for this is unknown.

1.5.5.b Ataxia

Amantadine reduces the functional disability of Friedrich's ataxia, an autosomal recessive disorder with progressive cerebellar dysfunction, dying back neuropathy, muscle weakness, scoliosis and hypertrophic cardiomyopathy. Its etiology is unknown (Peterson *et al.*, 1988).

1.5.5.c Brain Injury

Recovery from traumatic brain injury is known to be associated with neurobehavioural sequelea, these include low arousal, poor memory, attention and concentration deficits, emotional lability, agitation, irritability, assaultiveness, executive functioning deficits and depression. Several studies report that traumatic brain injury subjects exhibit some improvement in cognitive and/or physical function following treatment with amantadine (Nickels *et al.*, 1994).

1.5.5.d Creutzfeldt-Jakob Syndrome

Cruetzfeldt-Jakob disease is a virtually fatal brain disorder that may be viral in origin. Several reports have shown significant improvement in patients treated with amantadine (Parkes, 1974).

1.6 ADVERSE EFFECTS

Orally administered amantadine is generally well tolerated, and no serious renal, hepatic or hematopoietic toxicity has been documented. The most common side effects are minor gastrointestinal and central nervous system effects, including nervousness, light-headedness, difficulty in concentrating, insomnia, loss of appetite and nausea. Such effects relate to dose. Doses of 300 mg a day are associated with decreased psychomotor performance, such as diminished sustained attention spans and problem-solving abilities, whereas lower doses (100- 200 mg a day) are not associated with such changes. Among subjects receiving 200 mg a day for long term prophylaxis, 5 to 30 % had side effects. The side effects promptly disappear on discontinuation. Steady-state trough plasma concentrations of more than 0.45 μg/ml or peak concentrations of more than 1 μg/ml are associated with central nervous system effects and the incidence of such effects is increased by the concomitant ingestion of antihistamines and anticholinergic drugs (Greenblatt *et al.*, 1977, Oates *et al.*, 1990).

The long term use of amantadine has been associated with livedo reticularis, peripheral edema, orthostatic hypotension, and occasionally congestive heart failure, loss of

vision and urinary retention. Amantadine may increase seizure activity in patients with pre-existing seizure disorder. Amantadine is tetrogenic in rats (Greenblatt *et al.*, 1977), (Oates *et al.*, 1990).

1.7 WHY AMANTADINE WAS INVESTIGATED

Chloroquine, mefloquine and quinine are all lysosomotropic agents that accumulate to high levels in the food vacuole of the parasite and inhibit haemoglobin digestion in the parasite (Krogstad and Sclesinger, 1987). Amantadine by virtue of its weak base properties is expected to accumulate in the food vacuole of the parasite and may also exhibit anti-plasmodial activity.

CHAPTER TWO - BASIC METHODOLOGY

2.1 CULTURING *Plasmodium falciparum in vitro*

The *in vitro* cultivation of *P.falciparum* was carried out using a modification of the method of Freese *et al.*, 1988. The FCR-3 and RSA-2 strains were obtained from J. Freese of the Research Institute for Diseases in the Tropcial Environment, Durban. The 3D7A and 7G8 strains were obtained form D.Walliker, WHO Registry of Standard Strains of Malaria Parasites, University of Edinburgh, Scotland.

2.1.1 CULTURE MAINTENANCE

Thin blood smears were made daily, examined and cultures treated accordingly. Spent medium was aspirated off and replaced with fresh gassed medium. The flasks were sealed and incubated at 37°C. The FCR-3 strain was cultured under static conditions. All other strains were cultured on an orbital shaker (200 revolutions/min) to prevent the formation of sexual forms (gametocytes).

2.1.1.a Preparation of culture medium

RPMI-1640 medium was prepared as directed, this was supplemented with 20 mM glucose, 25 mM HEPES, hypoxanthine (44 mg/l) and gentamicin (50 mg/l). The medium was filtered, gassed with sterile CO_2 and stored at 4°C. Prior to use the medium was further supplemented with 10% human plasma and 5% $NaHCO_3$ to give a final pH 7.4.

2.1.1.b Preparation of plasma

Human plasma (AB^+) from at least three individuals was pooled. The plasma was heat inactivated at 56°C for 2 hours then centrifuged at 3000 rpm for 10 minutes to remove the debris, the supernatant was aliquoted out and stored at -70°C.

2.1.1.c Preparation of erythrocytes

Human red blood cells (O^+) stored in CPD-A were aliquoted out, washed twice (1500 rpm, 5 min) in PBS (pH 7.4), stored at 4°C and used within 48 hours.

2.1.1.d Assessment of parasite growth and development

A drop of cells was placed on a slide and a second slide dragged up to the drop so the drop formed a menisicus at the acute angle between both slides. A standard Giemsa staining procedure for thin smears was done using 10% Giemsa solution for about 20 min at room temperature. The percentage parasitemia was determined by counting the number of parsites in at least 1000 RBCs.

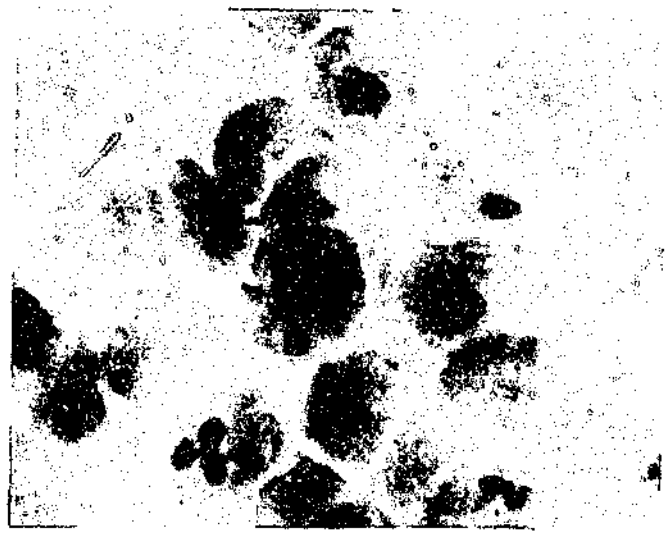


Figure 2.1: Typical thin blood smear

2.1.2 THAWING

The frozen vial was thawed, the contents transferred to a centrifuge tube and 0.1 ml 9% NaCl solution per 1 ml blood suspension was added dropwise whilst the tube was shaken. The tube was left to stand for 3-5 minutes, then 9 volumes of 1.6% NaCl solution was added to the same tube, mixed gently and then centrifuged at 1500 rpm for 5 minutes. The supernatant was removed. The pellet was gently resuspended in 9 volumes of a 0.2% glucose and 0.9% NaCl solution and centrifuged at 1500 rpm for 5 minutes. The supernatant was again removed. The pellet volume was adjusted to 1.0 ml using fresh red blood cells. Fresh medium was added, the culture was sealed and incubated at 37°C.

2.1.3 FREEZING

Cultures, mainly in the early ring stage, were centrifuged at 1500 rpm for 5 minutes. The spent medium was removed. Packed cells were mixed as a 1:1 dilution with sterile 28% glycerol in PBS. Approximately 0.5 ml of the parasite suspension was aliquoted out into cryotubes. Tubes were stored in liquid nitrogen.

2.1.4 PARASITE CONCENTRATION (Goodyer *et al.*, 1994)

The gelatin floatation technique was used to concentrate parastites ~10 fold to parasitemias of 50-70% - see figure 2.2. Swine skin gelatin (300 bloom) was dissolved in incomplete medium. Cultures containing mainly late trophozoites and schizonts (parasitemia > 8%) were centrifuged at 1500 rpm for 5 min to separate the erythrocyte from the culture medium, which was discarded. The erythrocytes were suspended in 1% gelatin solution (at 37°C) to give 10% haematocrit and aliquoted

into plastic tubes. The tubes were placed upright in a water bath at 37°C for approx 15 minutes. The suspension separated into two distinct phases : the supernatant phase contained erythrocytes infected with trophozoites and schizonts and the settled phase contained uninfected erythrocytes and any ring and early trophozoite stages. The supernatant phase was washed 3 times with incomplete medium and further processed according to experimental protocols. The pellet phase was discarded or returned to culture.



Figure 2.2: Trophs, before and after

2.1.5 PARASITE SYNCHRONIZATION

Plasmodium falciparum in culture loses the normal synchrony shown *in vivo* therefore it is necessary to synchronize cultures.

2.1.5.a Routine (Lambros and Vanderberg, 1979)

Cultures, mainly in the early ring stage, were centrifuged at 1500 rpm for 5 minutes. The spent medium was removed, the pellet was resuspended in 10 volumes of 5% D-sorbitol and allowed to stand for 15 minutes at room temperature before being

centrifuged at 1500 rpm for 5 minutes. The supernatant was removed and the pellet was transferred to a culture flask, to which fresh medium had been added. The culture was sealed and incubated as described before.

2.1.5.b Specific (Pfaller *et al.*, 1982)

The parasites were concentrated using the gelatin floatation method, the resulting supernatant phase consisting of late trophozoites and schizonts was placed in culture with fresh uninfected erythrocytes and allowed to incubate for 8-12 hours at 37°C. This allowed some of the schizonts to mature to merozoites and invade new erythrocytes. Following this incubation period, the culture was treated with 5% D-sorbitol as outlined above, thus destroying all stages but the ring stage. The sequential gelatin and sorbitol treatment results in a tight synchrony which is maintained for at least three cycles i.e. >144 hrs.

2.1.6 PARASITE ISOLATION (Wuderlich *et al.*, 1987)

Parasitized erythrocytes (parasitemia > 8%) were washed in incomplete medium and lyzed by incubation for 30 min at 37°C in 20 volumes of 0.015% solution of saponin prepared in medium - see figure 2.3. After low speed centrifugation, the pellet of parasites was recovered and the supernatant, which contained soluble material and erythrocyte ghosts, was discarded. The pellet was washed twice in medium. Approx 2.5 hours were required to isolate parasites. The parasite suspension was kept on ice and used within 2.5 hours.

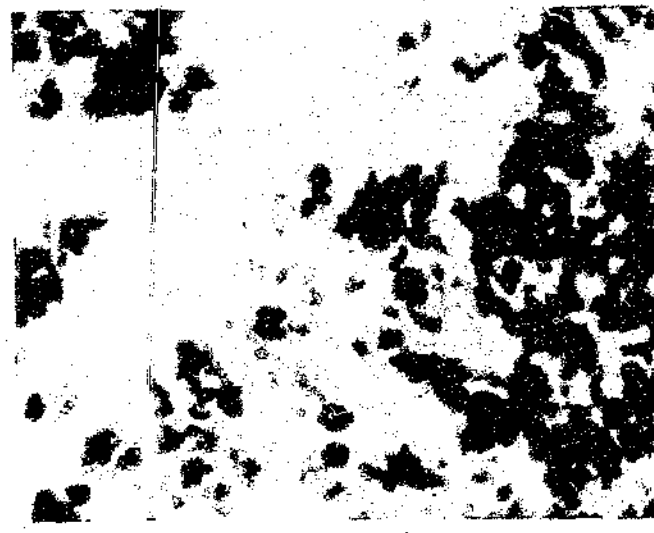


Figure 2.3: Isolated parasite

2.2 *In vivo* MODEL (Peters *et al.*, 1987)

2.2.1 INFECTION OF ANIMALS

2.2.1.a Mice

Female Balb/c mice, 6-8 weeks old, were obtained from colonies maintained at the Central Animal Services Unit, University of the Witwatersrand. All mice were housed in plastic cages, not more than five animals per cage, at a controlled temperature of ~22°C. A normal diurnal light cycle was maintained, animals were kept in quiet surroundings and received water and standard food *ad libitum*.

2.2.1.b Parasites

Plasmodium berghei obtained from J Bush, SAIMR was used. The parasites were kept frozen in liquid nitrogen as a 1:2 dilution of infected blood in 28% glycerol. The parasite was passaged once or twice in Balb/c mice before use in each experiment. Briefly, the frozen vial was thawed, donor mouse/mice received a single inoculum of 0.2 ml of parasite suspension intraperitoneally. Parasitemia was allowed to develop to

± 20 %, at which time blood was collected and used to inoculate subsequent mice - see figure 2.4

2.2.1.c Preparation of inoculum

The animal was anesthetized by intra-muscular injection of a droperidol-fentanyl combination (Innovar-vet). The limbs were secured onto a dissection board and the thorax was opened. Blood was collected from the dorsal vena carva, at the hepatic junction. The blood was spun at 1500 rpm for 5 min, the buffy coat of leukocytes and excess citrate was removed from the underlying erythrocytes using a Pasteur pipette with a flat tip. When more than one donor mouse was used the blood was pooled. The blood was diluted in mouse tonicity phosphate buffered saline so that each 0.2 ml contained approx 10^7 parasitized red blood cells.

2.2.1.d Infection

Recipient mice received a single inoculum of 0.2 ml, intraperitoneally. The day of infection was termed D0 and succeding days as D1, D2 etc. The course of the infection was monitored as described. On D8 mice were euthanased by placing mice in an environment saturated with CO_2.

2.2.2 MONITORING OF CLINICAL COURSE OF INFECTION

2.2.2.a Weight

The mice were weighed daily. A loss of >15 % total body weight was taken as an endpoint on ethical grounds.

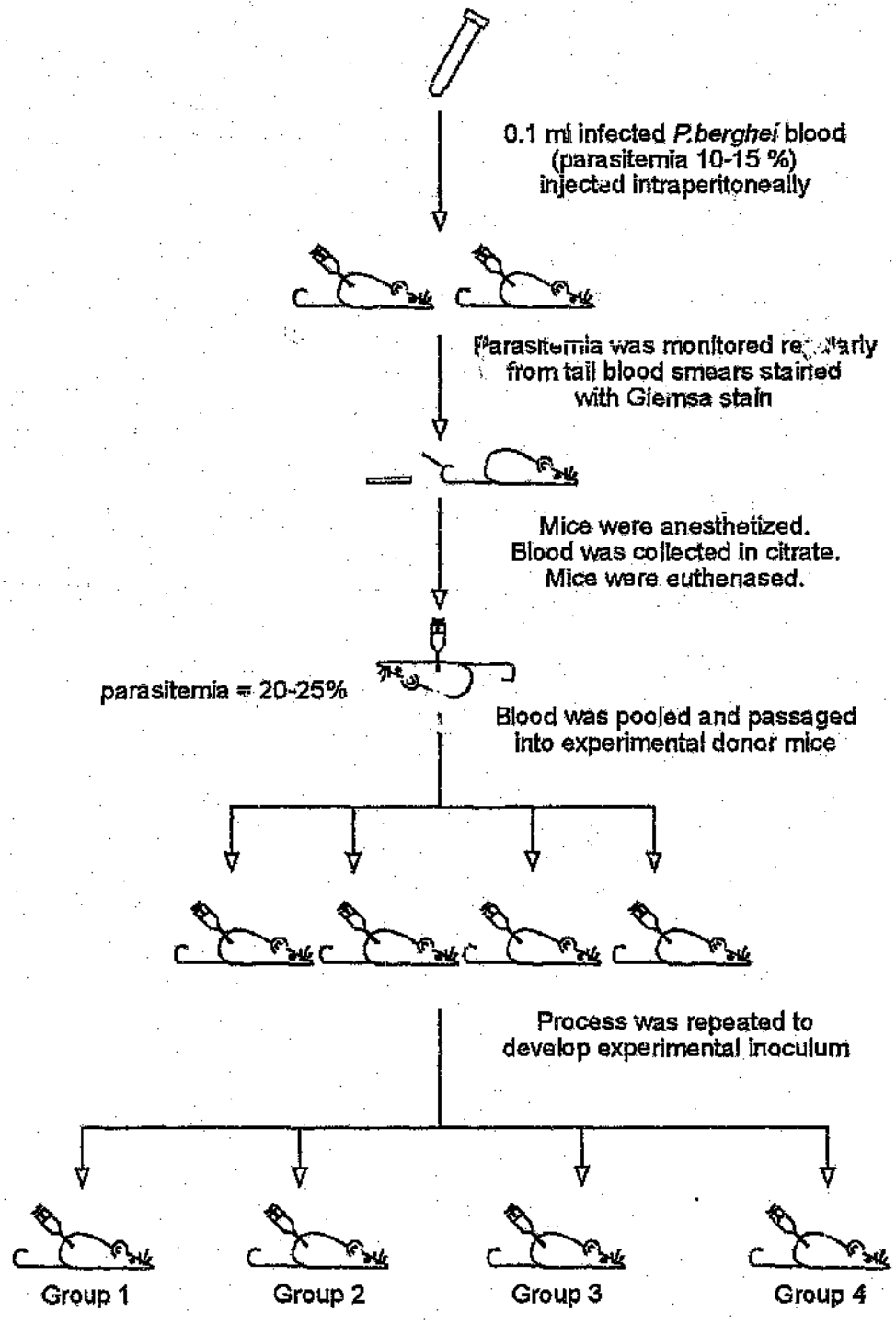


Figure 2.4 : Scheme of incoculum development

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CHAPTER THREE - DRUG SENSITIVITY

3.1 INTRODUCTION

Drug sensitivity tests are normally carried out *in vitro* against laboratory adapted strains of *P.falciparum* and/or *in vivo* against various species of rodent malaria since research involving the human host is sanctioned only in the final stages of drug trials.

In this section the IC_{50} values (concentration of drug causing a 50% inhibition of parasite growth) of a number of antimalarials as well as amantadine were determined for the local stains of *P.falciparum* and the influence of several variables on drug sensitivity *in vitro* were investigated. The *in vivo* activity of amantadine was assessed by monitoring the clinical course of *P.berghei* infection in Balb/c mice.

3.2 EXPERIMENTAL PROCEDURE *IN VITRO*

Parasite viability was assessed using a radiolabelled hypoxanthine method developed by Desjardins (1979).

3.2.1 PREPARATION OF PARASITES

Samples of routinely synchronised stock cultures (in the early ring stage) were diluted in hypoxanthine-free medium (prepared as described - omitting hypoxanthine and gentamicin) containing uninfected RBCs to yield a final haematocrit of 1% and parasitemia of 0.5%.

3.2.2 PREPARATION OF DRUGS

Drugs were freshly prepared, stock solutions were diluted to give the various concentrations using hypoxanthine-free medium. Ethanol concentrations never exceeded 1% of the final concentration, this concentration has previously been shown to have no significant effect on the malaria parasite. Drugs are arranged alphabetically.

3.2.2.a Amantadine

Amantadine hydrochloride was dissolved in water and filtered through a 0.22 µm filter.

3.2.2.b Chloroquine

Chloroquine sulphate was dissolved in water and filtered throuth a 0.22 µm filter.

3.2,2.c Halofantrine

Halofantrine was dissolved in 10% DMSO in water and filtered through a 0.22 µm filter. Halofantrine is very poorly soluble in water and at higher concentrations (> 1 mM) a precipitate forms in the wells.

3.2.2.d Mefloquine

Mefloquine was dissolved in 10% ethanol in water and filtered through a 0.22 µm filter.

3.2.2.e Primaquine

Primaquine was dissolved in water and filtered through a 0.22 µm filter.

3.2.2.f Pyrimethamine

Pyrimethamine was dissolved in water (by adjusting the pH ± 4 and stirring for 180 min on a magnetic stirrer) and filtered through a 0.22 μm filter.

3.2.2.g Quinine

Quinine was dissolved in water and sterilised by autoclaving at 121°C for 20 minutes.

3.2.3 ASSESSMENT OF PARASITE GROWTH

3.2.3.a Preparation of microtitre plates

The parasites (200 μl of a suspension with initial parasitemia of 0.5% and haematocrit of 1%) were incubated in a 96-well flat bottomed microtitre plate in hypoxanthine-free medium, in the presence of 25 μl of drug appropriately diluted (each drug concentration was in triplicate). The plates contained a negative control (4 wells with non-parasitized RBCs, no drug) and a positive control (8 wells with parasitized RBCs, no drug) as shown in figure 3.1. The plates were placed in a candle jar. An anaerobic environment was created by burning oxygen. The plates were incubated at 37°C for 24 hours.

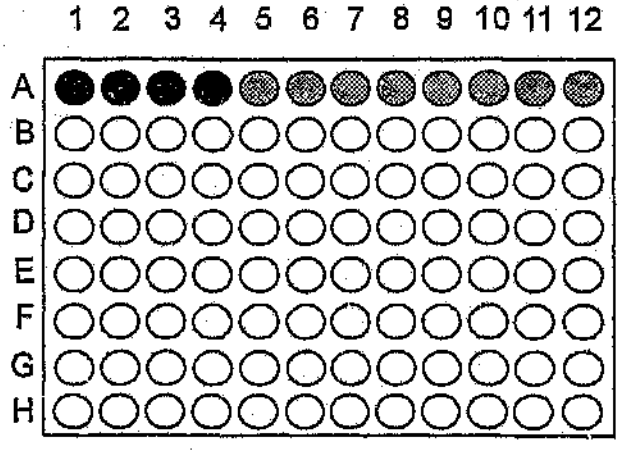


Figure 3.1 : Schematic diagram of 96-well plate. Black wells negative control, grey wells positive control and white wells experimental

3.2.3.b Preparation of isotope

The contents of a single ampoule was dissolved in 2 ml 50% ethanol and stored at -20°C forming the stock solution. The appropriate volume of isotope stock solution was aliquoted out into a glass tube. The ethanol was evaporated using a steady stream of sterile gas. Hypoxanthine-free medium was added to the isotope/water mixture to give the correct final volume.

3.2.3.c Labelling the parasites

1.85 μCi (25 μl) of isotope mixture was added to each well. The plates were returned to the candle jar and incubated for a further 18 hours at 37°C.

3.2.3.d Harvesting parasites

The plates were harvested by aspirating and depositing the contents of each well onto a small disk of filter paper and washing with copious volumes of distilled water (Flow Laboratory Titertek Cell Harvester). Disks were air dryed and placed in glass vials to which 5 ml of scintillation fluid (Aquagel) was added. Vials were counted in scintillation spectrometer for 1 minute.

3.2.3.e Data analysis

Counts were corrected for incorporation of [^{3}H]-hypoxanthine into uninfected and non-drug treated cells. IC_{50} values (representing the molar concentration resulting in 50% decrease in [^{3}H]-hypoxanthine incorporation compared to drug free controls) were obtained for each drug using a computer programme Enzfitter®, Biosoft, UK (based on Marquart's algorithm) using the equation :

$$\% \text{ parasite survival} = \frac{100}{1+(C / IC_{50})^{p}}$$

where C = concentration of the drug

IC_{50} = 50 % inhibitory concentration of the drug

p = slope

3.3 EXPERIMENTAL PROCEDURE *IN VIVO* (Peters *et al.*, 1987)

3.3.1 INFECTION OF ANIMALS

Parasite inoculum was developed as outlined in figure 2.5. The clinical course of infection was monitored.

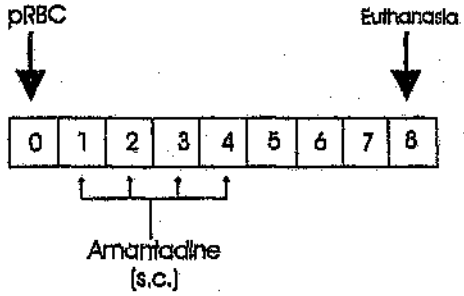
3.3.2 TREATMENT

3.3.2.a Drug preparation

Amantadine hydrochloride was diluted in 0.9 % NaCl to give the dose required in 0.1 ml for 10 g mouse and filtered and was administered subcutaneously

3.3.2.b Drug administration

All experimental mice were infected at random before being divided into groups of five mice. Drugs were administered according to the schedule indicated.



3.4 RESULTS

3.4.1 DOSE RESPONSE *in vitro*

3.4.1.a Standard antimalarials

A typical hypoxanthine derived dose response graph illustrating the response of the FCR-3 strain parasites to amantadine in a single experiment is shown in figure 3.2 and 3.3. An excellent fit of the data to the regression equation is obtained; the IC_{50} for this experiment was 5.44 μM with a 95 % confidence interval of 5.25 to 5.62 μM and a slope of -1.3.

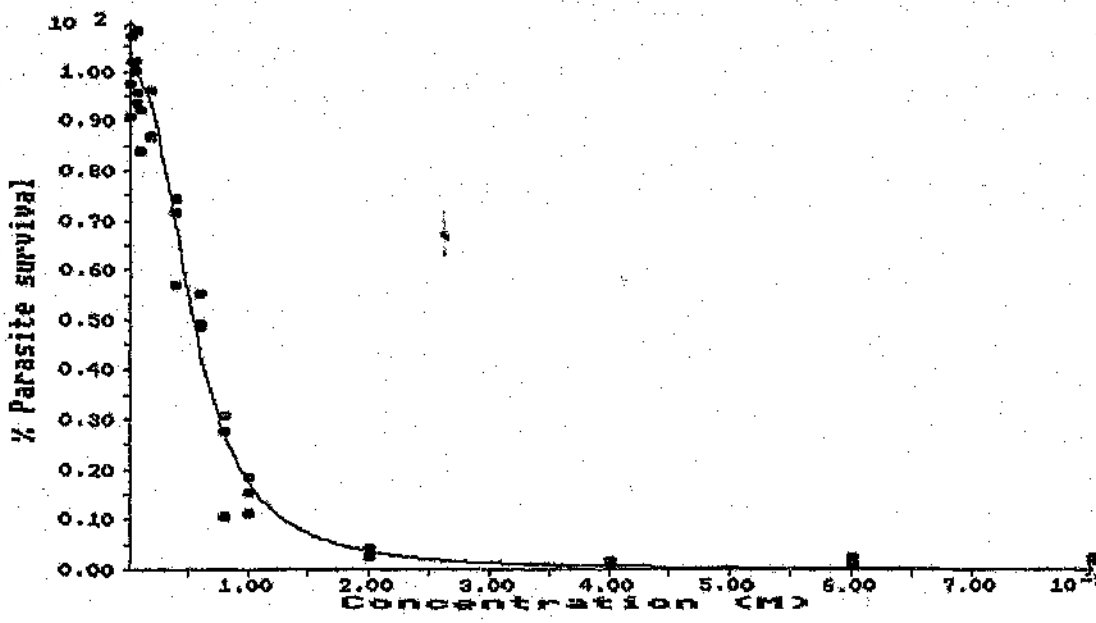


Figure 3.2: Dose response curve amantadine against the FCR-3 strain of *P.falciparum in vitro*

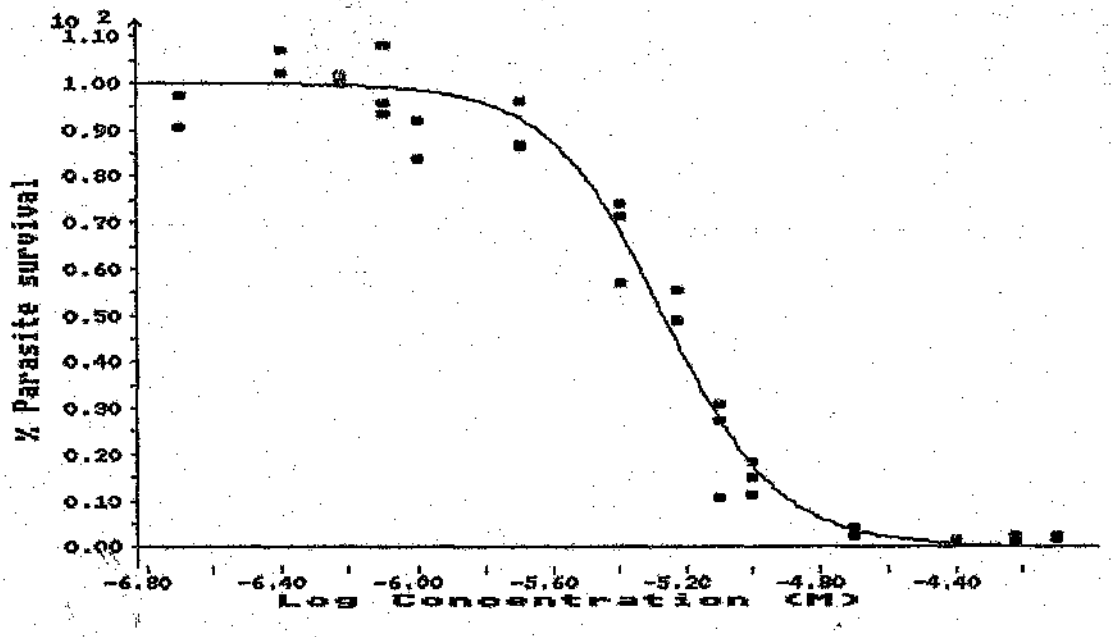
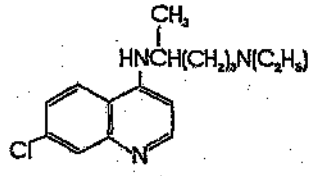
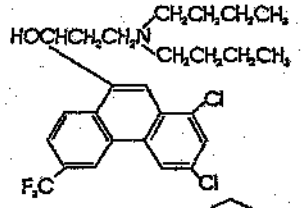
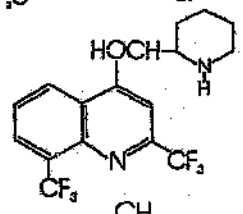
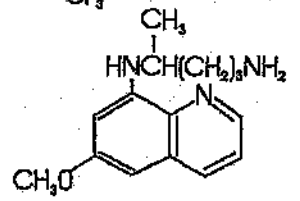
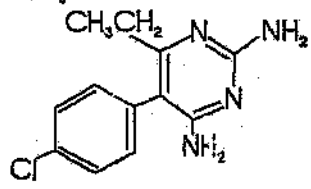
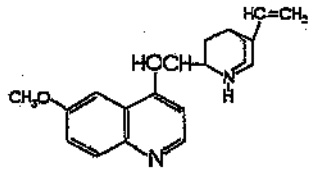


Figure 3.3 ; Log transformation of dose response curve of amantadine against FCR-3 strain of *P.falciparum in vitro*

Table 3.1 shows how two strains of *Plasmodium falciparum*, FCR-3 and 3D7A, differ in their sensitivities to standard antimalarials. The degree of resistance is reflected by the ratio of IC_{50} of the FCR-3 strain to the IC_{50} of the 3D7A strain. The FCR-3 strain is resistant to chloroquine, quinine and pyrimethamine as reflected by a ratio >1, but shows increased sensitivity to mefloquine, halofantrine and primaquine as indicated by a ratio < 1 when compared to 3D7A strain. The values are means of 6-15 determinations.

Table 3.1 : Potencies of standard antimalarials against two strains of *Plasmodium falciparum in vitro*

DRUG	STRUCTURE	FCR-3 (nM)	3D7A (nM)	RATIO FCR-3/3D7A
Chloroquine		176.4 ± 2.74	2.01 ± 0.04	87.76
Halofantrine		0.8 ± 0.68	6.26 ± 0.26	0.13
Mefloquine		15.4 ± 1.21	38.8 ± 1.53	0.40
Primaquine		1300 ± 60	4150 ± 180	0.31
Pyrimethamine		149 ± 5.43	5.38 ± 1.53	2.78
Quinine		141 ± 3.57	64.9 ± 3.43	2.17

3.4.1.b Amantadine

Table 3.2 shows the antimalarial effect of amantadine against four strains of *Plasmodium falciparum*. The slope of the dose response curves does not differ

significantly between strains. Antimalarial activity ranges from 5 - 300 μM, the greatest activity is associated with chloroquine-resistant strains (FCR-3 and 7G8).

Table 3.2 : IC_{50} values for amantadine and the slope for different strains of *P.falciparum*

STRAIN	IC_{50} Amantadine (μM)	SLOPE
FCR -3	5.35 ± 1.15	-1.72 ± 0.17
7G8	33.72 ± 6.2	-1.39 ± 0.15
RSA-2	188.44 ± 11.3	-2.22 ± 0.23
3D7a	297.82 ± 7.5	-1.81 ± 0.21

A comparison of amantadine sensitivity to chloroquine sensitivity is plotted in figure 3.4, revealing a unique relationship between the two drugs. Sensitivity to chloroquine decreases exponentially with increasing amantadine sensitivity (r = 0.995). Mefloquine is also generally more active against chloroquine-resistant strains than sensitive strains. We examined the chloroquine-mefloquine sensitivity (fig 3.5) and mefloquine-amantadine sensitivity (fig 3.6) relationship in the same four laboratory adapted strains. Chloroquine sensitivity was negatively correlated with mefloquine sensitivity (r = 0.949) as previously reported (Winkler *et al.*, 1994). Mefloquine and amantadine sensitivity were positively correlated (r = 0.82).

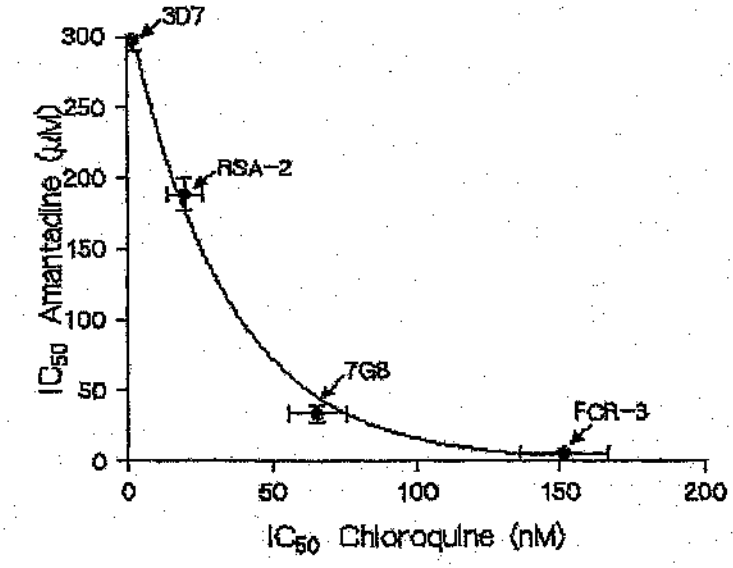


Figure 3.4 : Comparison of amantadine and chloroquine sensitivity in different strains of *P.falciparum*

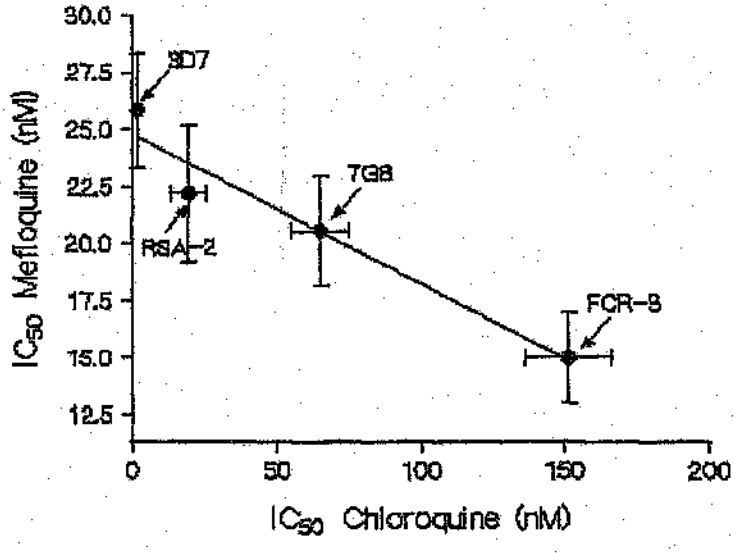


Figure 3.5 : Comparison of mefloquine and chloroquine sensitivity in different strains of *P.falciparum in vitro*

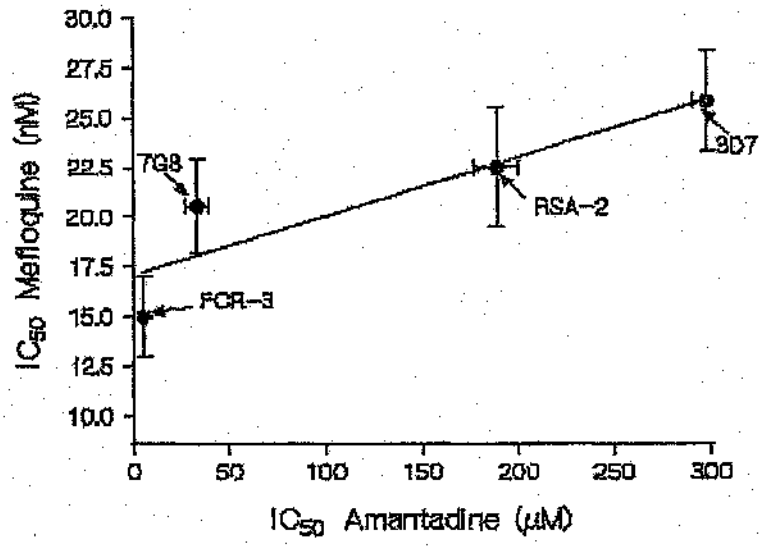
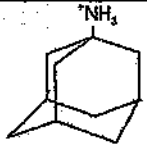
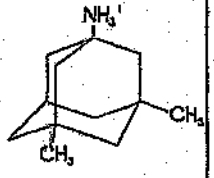


Figure 3.6 : Comparison of amantadine and mefloquine sensitivity in different strains of *P.falciparum in vitro*

The shape and size of the hydrocarbon cage and the hydrophilic group are exceedingly important for anti-Parkinson and antiviral activity of amantadine (Warnick *et al.*, 1982). The three dimensional components critical for activity differs : memantine is most potent in cerebral disorders while rimantadine is most effective as an antiviral. The effect of memantine (kindly donated by Merz Co, Germany) was compared to that of amantadine using radiolabelled hypoxanthine uptake method - see table 3.3. Memantine has C-alkyl substitutions on two carbons, carbon 3 and 5. The di-substitutions increased the antimalarial activity against both strains. The increase in activity was greatest against the chloroquine-resistant strain: memantine was ~3.5 fold more active against the chloroquine-resistant strain and ~2.2 fold more active against the chloroquine-sensitive strain.

Table 3.3 : Comparison of antimalarial activity of aminoadamantanes against a chloroquine-resistant (FCR-3) and a chloroquine-sensitive (3D7a) strain of *P.falciparum*

COMPOUND	STRUCTUR	IC_{50} / µM CQ-R (FCR-3)	IC_{50} / µM CQ-S (3D7a)	RATIO CQ-S/CQ-R
Amantadine		6.27 ± 0.44	230.71 ± 15.71	36.8
Memantine (Mrz 2/145)		1.78 ± 0.15	102.88 ± 3.08	57.8

3.4.2 FACTORS INFLUENCING AMANTADINE SENSITIVITY *IN VITRO*

The conditions under which the parasite is exposed to drug varies considerably between the *in vitro* and *in vivo* situations. For example, in *in vitro* tests the blood is diluted which lowers haematocrit, this affects the test endpoint for drugs which concentrate in the blood cells e.g. mefloquine (Vanderkooi *et al.*, 1988). Furthermore, where potent ligands are present in blood (e.g. acute phase proteins like α_1-acid-glycoprotein) diluted blood will present a changed ratio of free/bound drug to parasitized erythrocyte, in the case of quinine this affects the test endpoint (Birley *et al.*, 1992). The presence of humoral and cellular immune factors in test system in semi-immune patients artificially lowers the test endpoint. The influence of several variables on amantadine sensitivity were examined.

3.4.2.a Influence of inoculum size

Parasitized erythrocyte suspension was aliquoted to give varyir starting parasitemia and haematocrits and drug sensitivity examined. Dose re onse curves for the different inoculum sizes were calculated and the IC_{50} was plo ed against the product of haematocrit and parasitemia. Figure 3.7 and 3.8 show the e ct of inoculum size on amantadine and chloroquine sensitivity respectively in a c oroquine-resistant and chloroquine-sensitive strain. Sensitivity to amantadine was di ctly proportional to the number of parasites present in the chloroquine-sensitive (3D a) strain with a slope of 11.71 (r = 0.965), however, inoculum size has little effect or amantadine sensitivity in the chloroquine-resistant (FCR-3) strain with a slope of 0.23

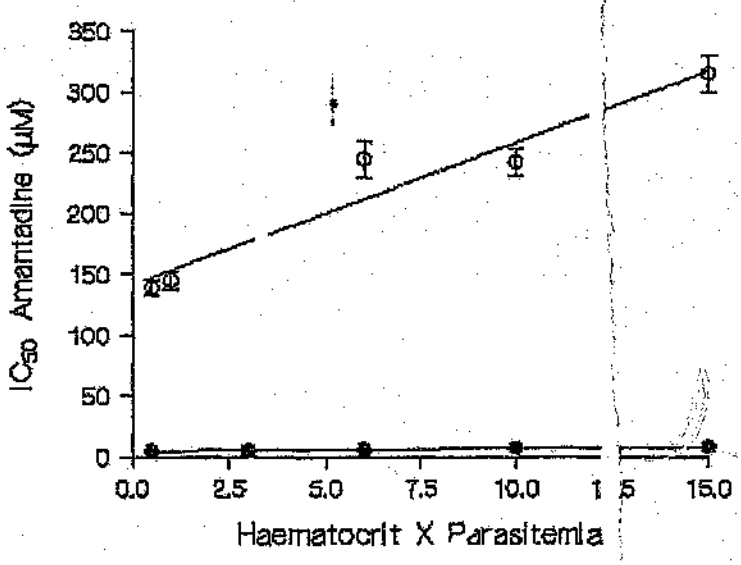


Figure 3.7 : Inoculum size versus IC_{50} of amantadine against chlor uine-resistant (●) and chloroquine-sensitive (O) strains of *P.falciparum*

Chloroquine-sensitivity was directly proportional to inoculum size in both the chloroquine-resistant and chloroquine-sensitive strains with the slope being 18.69 (r = 0.901) and 7.0164 (r = 0.92) respectively, in agreement with results previously published (Geary *et al.*, 1990).

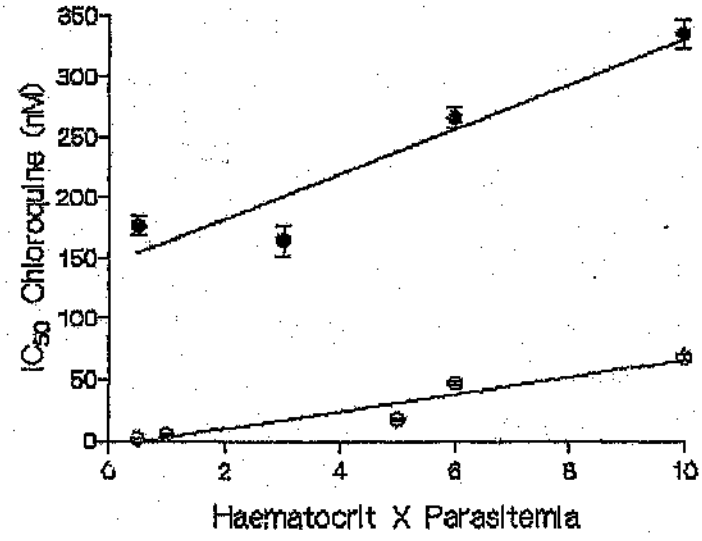


Figure 3.8 : Inoculum size versus IC_{50} chloroquine against chloroquine-resistant (●) and chloroquine-sensitive (○) strains of *P.falciparum*

3.4.2.b Influence of plasma concentration

The IC_{50} of amantadine in the presence of 10% plasma (normal concentration *in vitro*) was compared to the IC_{50} in the presence of 2% plasma (minimum level required to sustain parasite growth). Figure 3.9 shows amantadine inhibition is not reduced by the presence of plasma in the extracellular medium, implying that amantadine is not binding to plasma components.

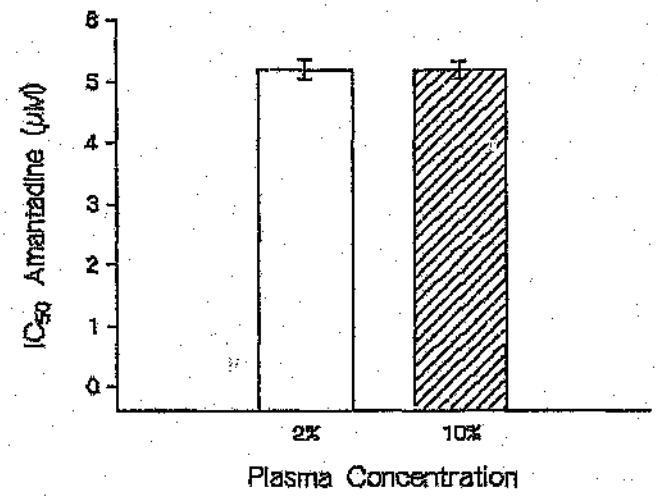


Figure 3.9 : Comparison of IC_{50} of amantadine at different concentrations of plasma

3.4.3 DOSE RESPONSE *IN VIVO*

Groups of five mice were injected s.c. daily (from day 1 to day 4 of infection) with 0, 5, 10 or 25 mg/kg amantadine hydrochloride suspended in saline. A significant dose-dependent shift towards the right in the course of parasitemia was observed (fig 3.10).

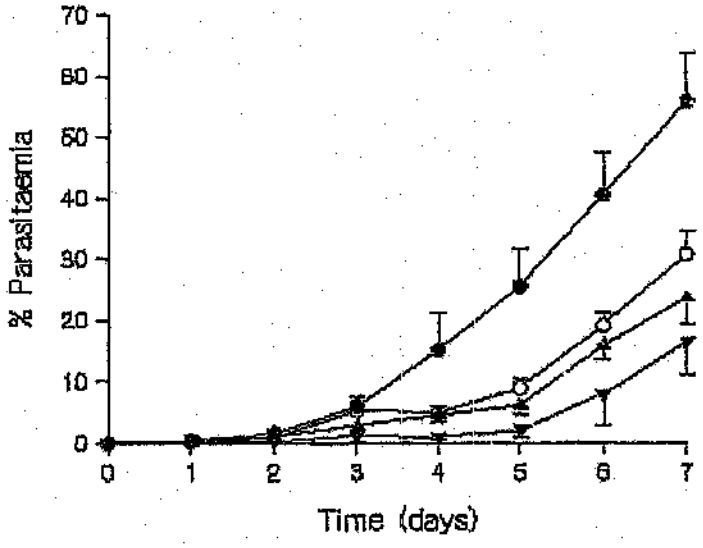


Figure 3.10: Dose response of amantadine against *P.berghei* (●) Control (○) 5 mg/kg (▲) 10 mg/kg and (▼) 25 mg/kg

The decrease in the parasitemia was accompanied by a decrease in morbidity as seen with weight (results not shown) and haematocrit (a measure of anaemia) (fig 3.11). All infected mice showed significant anaemia, the haematocrit being significantly lower than ± 48 % found in healthy age matched controls. The haematocrit in amantadine treated groups was significantly higher ($p < 0.01$) than in the control group. This difference probably reflects the lower parasitemia. Haematocrit is closely correlated with the level of parasitemia.

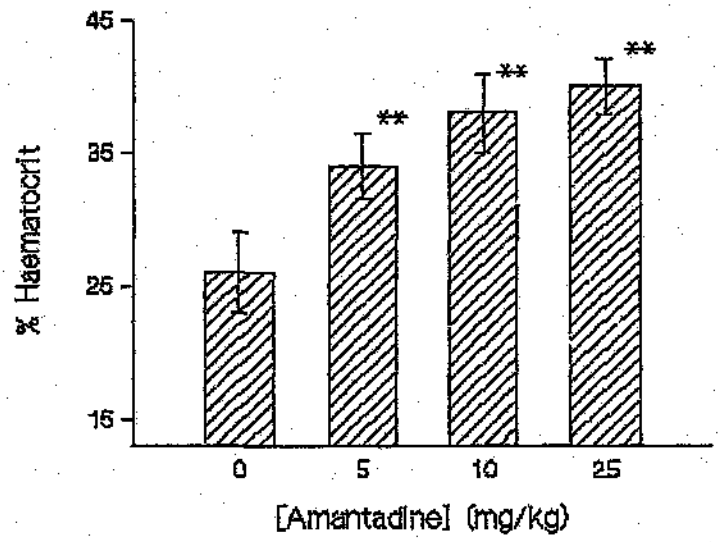


Figure 3.11: Haematocrit on day 7 of *P.berghei* infection in Balb/c mice.

Amantadine failed to clear the parasites. Once administration of the drug ceased on day 4 the parasitemia started to rise. The growth rate was similar to that seen in the untreated group, the slight decrease reflecting the residual effect of amantadine rather than true difference (fig 3.12). The effect of amantadine against *P.berghei* was cytostatic rather than cytocidal.

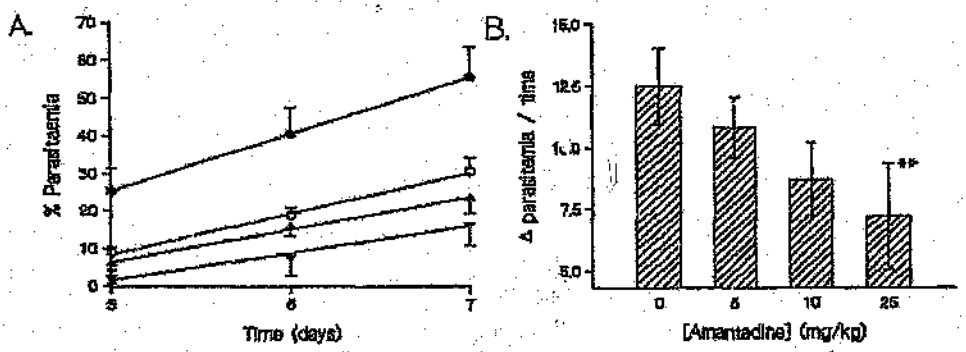


Figure 3.12: Rate of increase of parasitemie after drug administration (day 5 +) (●) Control (○) 5 mg/kg (σ) 10 mg/kg and (τ) 25 mg/kg (** $p < 0.05$)

3.5 DISCUSSION

Amantadine displays antimalarial activity both *in vitro* and *in vivo*. The concentrations of amantadine exhibiting antimalarial activity against chloroquine-resistant strains of *P.falciparum in vitro* are attainable *in vivo* in man: oral administration of 100 mg twice daily results in steady-state concentrations of 0.1-1.1 μg/ml (0.6-7.3 μM) (Greenblatt *et al.*, 1977). Amantadine sensitivity has a distinctive relationship with chloroquine-resistance: amantadine sensitivity is exponentially correlated with chloroquine sensitivity (fig 3.4). A similar phenomenon has been documented between chloroquine and mefloquine (Cowman *et al.*, 1994, Winkler *et al.*, 1994) (see figure 3.5), however, the magnitude of the effect is substantially greater with amantadine (resistance ratio = 0.02) compared with mefloquine (resistance ratio = 0.4). We have coined the term "inverse resistance" to describe this new category of drugs (Evans and Havlik, 1994).

The concept of "inverse resistance" drugs is new. Combinations of such agents, with chloroquine could provide an additional strategy for combating chloroquine resistant malaria while retaining chloroquine as part of the arsenal of drugs effective in malaria therapy. Despite the widespread resistance to chloroquine it remains the drug of choice due to its low cost, rapid onset of action and low toxicity.

The *in vivo* activity of amantadine was disappointing (fig 3.10). Amantadine inhibited parasitemia and prolonged survival at all concentrations tested. Once administration of the drug ceased (day 5 +) the parasitemia developed as normal. These results suggest that should amantadine be used clinically, it would need to be given in combination with other antimalarials and administration would need to be continued for a longer period than traditional antimalarials. This strategy has proved to be effective for the use of antibiotics in malaria (Yeo and Rieckmann, 1994). In addition, the *in vivo* results of amantadine are complicated by the "inverse resistance" phenomenon. The strain of *P.berghei* used was chloroquine-sensitive - since amantadine sensitivity appears to be connected to chloroquine-resistance in *P.falciparum*, antiplasmodial activity is expected to be negligible in chloroquine-sensitive strains. Unfortunately, it was not possible to investigate this further since chloroquine-resistance in *P.berghei* appears to be different from that seen in *P.falciparum* (Walliker, personal communication). The chloroquine-resistant *P.berghei* also accumulate less chloroquine than sensitive parasites (Macomber *et al.*, 1966), as seen in *P.falciparum*. However,

the chloroquine-resistant strains of *P.berghei* preferentially invade and grow within reticulocytes. Growth in reticulocytes reduces the weak-base trapping of chloroquine irrespective of chloroquine sensitivity (Howells *et al.*, 1968, Slomianny *et al.*, 1984).

The inverse resistance relationship between amantadine and chloroquine is difficult to reconcile mechanistically but probably is connected to the mechanism(s) mediating chloroquine-resistance. The only consistent feature which distinguishes sensitive from resistant parasites is the level of chloroquine accumulation within the trophozoite (Geary *et al.*, 1986). The mechanism of resistance to quinoline-containing antimalarials is probably unrelated to its mode of action but related to a decrease in concentrations of drug in the parasite. This could take the form of enhanced chloroquine export (Krogstad *et al.*, 1987), reduced chloroquine uptake (Ferrari and Cutler, 1991, Ginsburg and Stein, 1991) or a combination of both.

Characterisation of the antimalarial activity of amantadine *in vitro* suggests that amantadine activity against chloroquine-resistant strains involves factors in addition to a concentration shift. Although the slope of the dose response curves does not differ significantly between the strains (table 3.2), the response to changes in the size of the inoculum vary between the strains (fig 3.7).

The food vacuole is believed to be the target of the classical antimalarials (Zarchin *et al.*, 1986) therefore, the intracellular concentration of the drug is critical for drug sensitivity. An increase in the number of parasites is expected to result in depletion of the extracellular drug, therefore an increased extracellular concentration of drug is needed to elicit the inhibitory effect. Amantadine sensitivity like chloroquine sensitivity is dependent on inoculum size against the chloroquine-sensitive strain (3D7a) (Geary *et al.*, 1990). However, the magnitude of this effect is very small (slope 0.23) in the chloroquine-resistant strain (FCR-3), this may be indicative of factors other than the concentration of the drug in the food vacuole being important in the antimalarial effect against the chloroquine-resistant strains.

The remainder of the thesis will examine the effect of amantadine in combination with other antimalarials. The developmental, lysosomotropic, membrane and reversal effects of amantadine will be studied to further define the inverse resistance phenomenon.

3.6 SUMMARY

Amantadine exhibits antimalarial activity both *in vitro* and *in vivo*. The activity of amantadine *in vitro* is more selective towards the chloroquine-resistant strains of *P.falciparum* (IC_{50} = 5 μM) - we have termed this inverse resistance. These antimalarial properties are exhibited at levels that are attainable in man (0.6-7.3 μM). Amantadine sensitivity is independent of inoculum size and plasma concentration.

CHAPTER FOUR - DRUG COMBINATIONS

4.1 INTRODUCTION

The current pharmacological approach to resistant malaria involves a number of strategies:

i) the development of new drugs (Peters *et al.*, 1993)

ii) drug combinations of classical antimalarials

iii) combinations of classic antimalarials and antibiotics (Gingras and Jensen, 1993)

iv) combinations of chloroquine and agents which "reverse" resistance, e.g. calcium channel blockers (Kyle *et al.*, 1990), tricyclic antidepressants (Bitonti *et al.*, 1988) and antihistamines (Peters *et al.*, 1990)

v) combinations of classic antimalarials and adjunct therapy (drugs that do not necessarily clear parasitemia, but play a crucial role in bringing parasitemia levels under control during the acute stages of infection), e.g. iron chelators (Hershko *et al.*, 1992) and heparin (Kulane *et al.*, 1992)

The level of activity of amantadine means that it is unlikely to be considered sufficient to support the use of the drug as a single entity. If amantadine were used clinically, it is most likely to be administered either simultaneously or sequentially with other antimalarials as an adjunct therapy. This component of the study investigates the

effect of amantadine in combination with several currently employed antimalarials and biochemical tools on the viability of *P.falciparum* as determined by isobologram analysis.

4.2 EXPERIMENTAL PROCEDURE

Stock solutions were prepared as described previously and drugs were mixed in fixed ratios (Berenbaum, 1978). The respective dose response curves and IC_{50}s for each drug ratio were determined from serial dilutions carried out in triplicate using Enzfitter Software®. Similar serial dilutions of the individual drugs were performed at the same time. Parasite growth was assessed using the hypoxanthine method. Isoboles in which the combination is represented by a point on a graph of the axes of the individual agents were constructed from the IC_{50} values of the combination dose response curves according to the equation:

$$\frac{d_a}{D_a}+\frac{d_b}{D_b} = \text{combination index}$$

where d_a and d_b = IC_{50} of agent A combined with agent B

D_a and D_b = IC_{50} of agent A and B alone respectively

If the agents do not interact (zero-interaction), the combination index will be equal to 1. If the effect of the combination is greater than expected (synergistic) the combination index will be < 1. If the combined effect is less than expected (antagonsitic) the combination index will be > 1.

4.3 RESULTS

The procedure to derive a isobologram is demonstrated. Figure 4.1 shows the dose response curves generated for the different ratios of chloroquine and amantadine against the RSA-2 strain. The combined IC_{50} values and the calculated combination index values are tabulated in table 4.1. The resulting isobole is plotted in figure 4.2. The superimposed solid line indicates the locus of all combinations that would have produced the effect if there had been no interaction, points below the line are synergistic and above the line antagonsitic.

Figure 4.3-4.7 shows the isoboles representing the interaction of amantadine with different classes of antimalarials on a chloroquine-resistant (FCR-3) strain and a chloroquine-sensitive strain (3D7a). The combination dose response curves for these combinations are not shown. IC_{50} values had standard errors of less than 10% of the mean. Experiments were performed in triplicate.

Figure 4.3 shows the interaction of amantadine with quinolines, the most commonly used group of antimalarials. Four quinolines were studied in detail: chloroquine, mefloquine, quinine and halofantrine. Amantadine was found to be slightly synergistic against the chloroquine-resistant strain (FCR-3), with all quinolines investigated. In the chloroquine-sensitive strain (3D7a) it was synergistic with quinine and chloroquine, but zero-interactive with mefloquine and halofantrine.

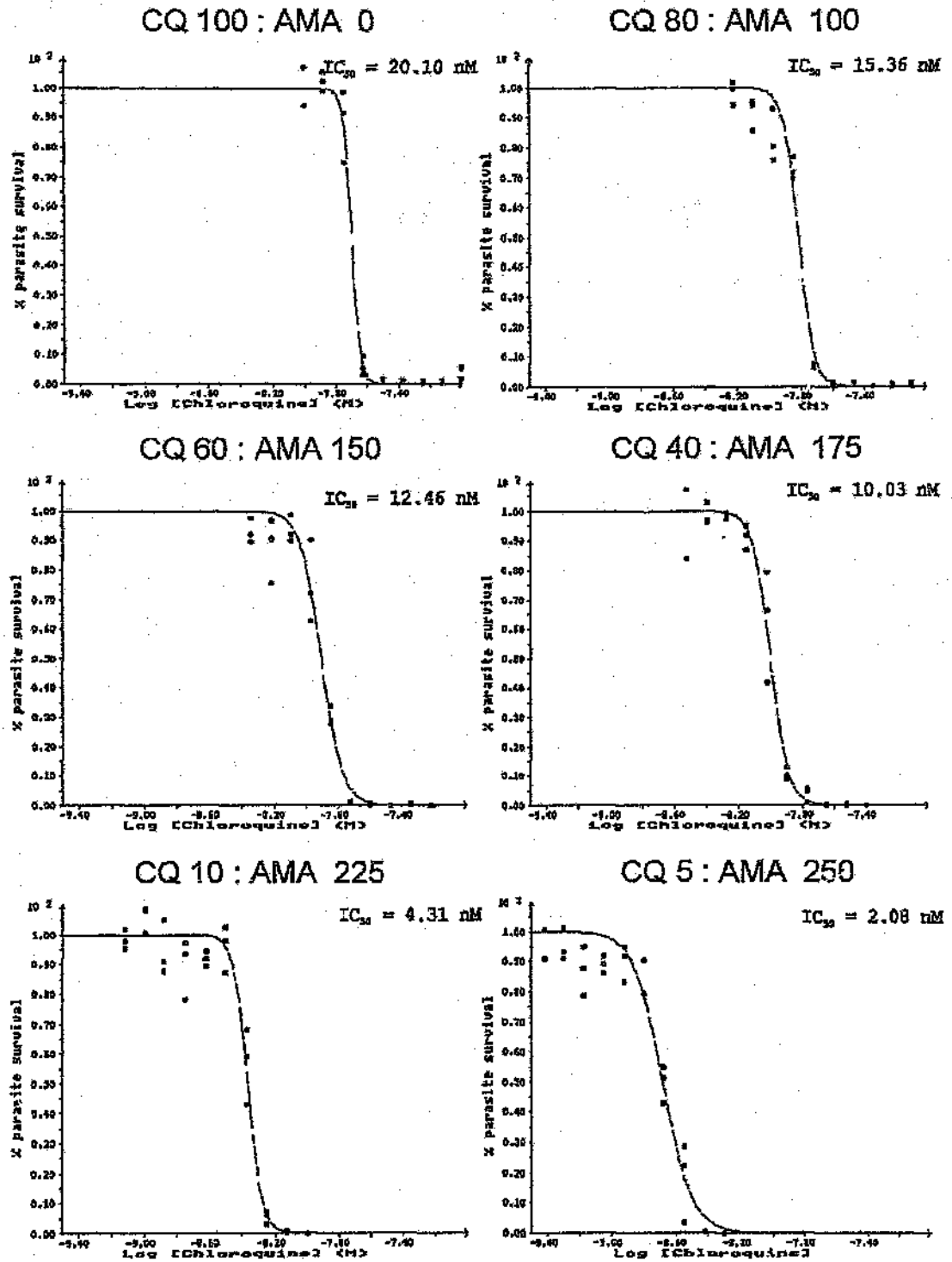


Figure 4.1: Dose response curves for chloroquine in the presence of amantadine against the RSA-2 strain of *P.falciparum*. The ratio of drugs is indicated at the top of each curve. The IC_{50} for chloroquine is shown in the top right hand corner of the graphs.

Table 4.1: Chloroquine-amantadine combination dose response curves for RSA-2 strain

Combination ratio	IC_{50} combination dose response		Ratio (d/D)		Combination index
CQ : AMA	CQ (nM)	AMA (μM)	CQ	AMA	CI
0 : 300	0	196.50	0	1.0	1.0
5 : 250	2.08	104.0	0.10	0.53	0.63
10 : 225	4.31	96.98	0.22	0.49	0.71
40 : 175	10.03	43.88	0.50	0.22	0.72
60 : 150	12.45	31.13	0.62	0.16	0.78
80 : 100	15.36	19.2	0.76	0.09	0.85
100 : 0	20.10	0	1.0	0	1

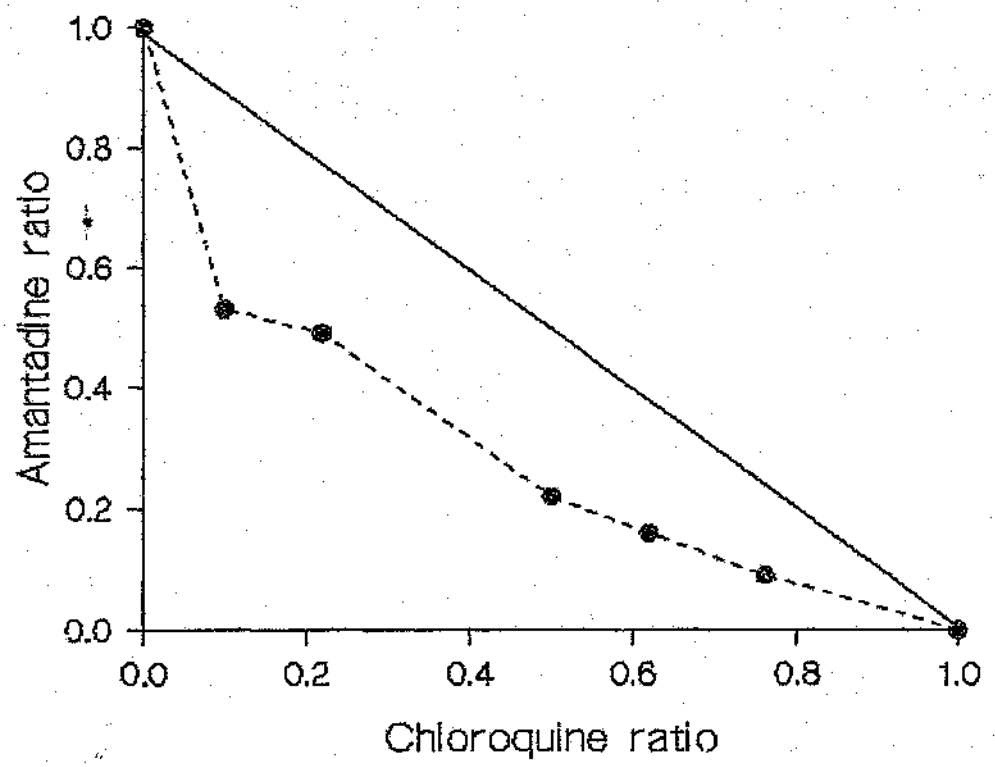


Figure 4.2: Isobologram depicting the interaction of amantadine with chloroquine against a chloroquine-sensitive (RSA-2) *Plasmodium falciparum* strain

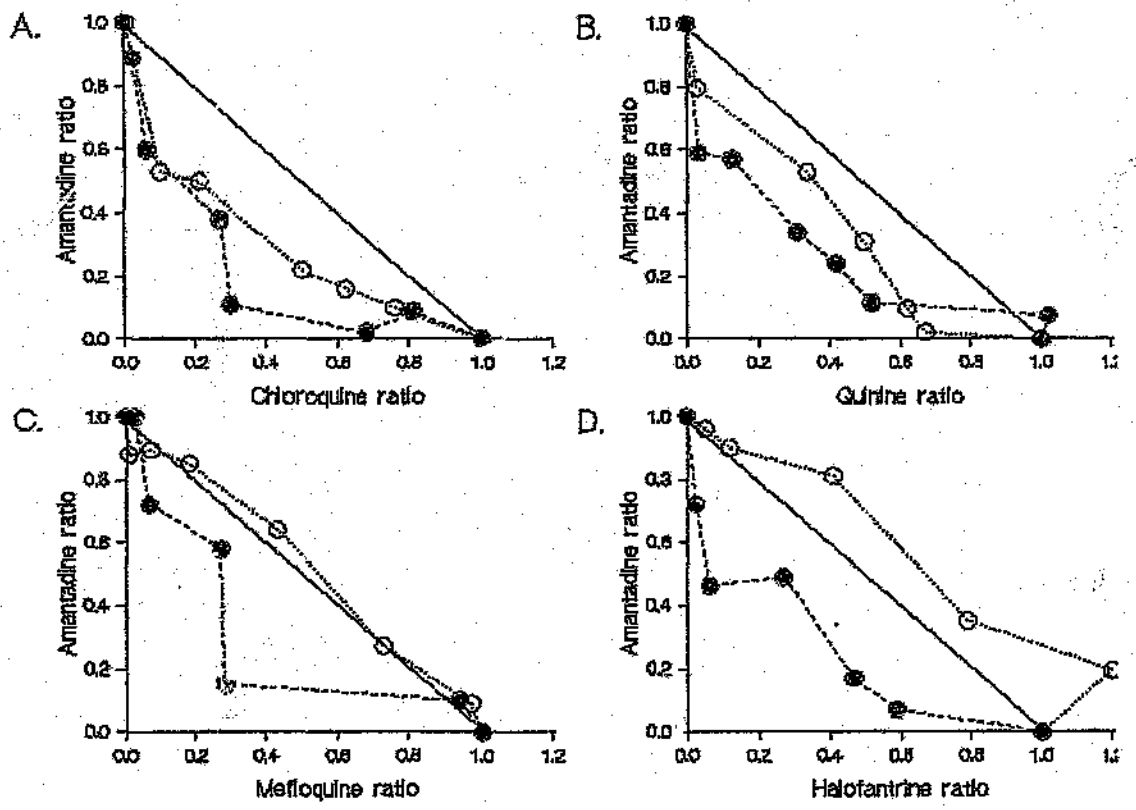


Figure 4.3: Isobolograms depicting the interaction of amantadine with quinolines. A. chloroquine, B. quinine, C. mefloquine and D. halofantrine against the chloroquine-resistant (●) and chloroquine-sensitive (O) strains of *P.falciparum in vitro*

The 8-aminoquinoline investigated, primaquine, was weakly synergistic in chloroquine-resistant (FCR-3) strain and zero-interactive in chloroquine-sensitive (3D7a) strain as seen in figure 4.4.

Figure 4.5, demonstrates the interaction of amantadine with the dihydrofolate reductase inhibitor, pyrimethamine. The interaction was zero-interactive irrespective of chloroquine sensitivity.

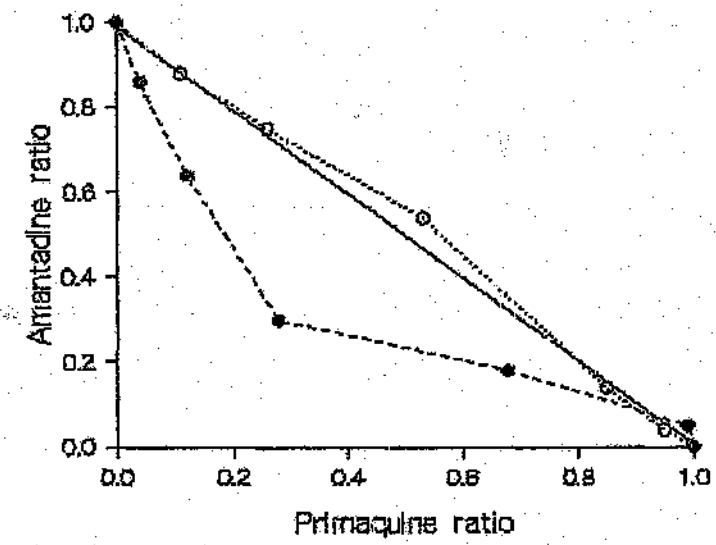


Figure 4.4: Isobologram depicting the interaction of amantadine with primaquine (8-aminoquinoline) against the chloroquine-resistant (●) and chloroquine-sensitive (O) strains of *P.falciparum in vitro*

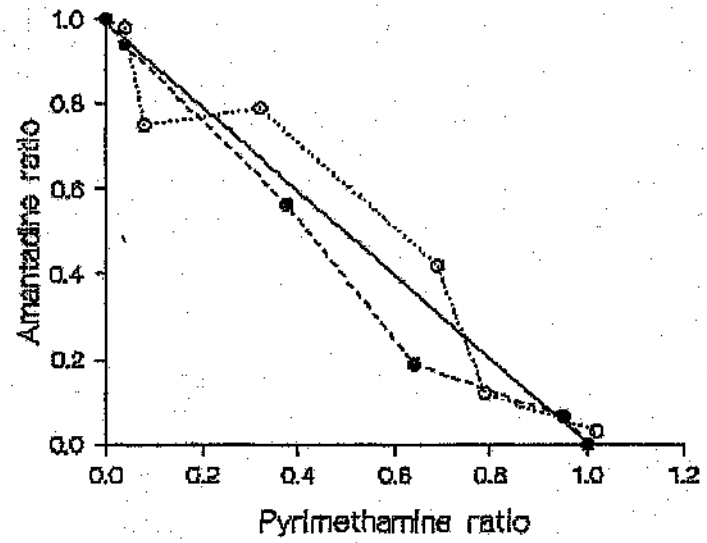


Figure 4.5: Isobologram depicting the interaction of amantadine with pyrimethamine against the chloroquine-resistant (●) and chloroquine-sensitive (O) strains of *P.falciparum in vitro*

A variety of antibiotics with distinctive mechanisms of action in prokaryotes, inhibit the survival of *P.falciparum in vitro* (Geary and Jensen, 1983), and *in vivo* either alone or in combination with other drugs (Andersen *et al.*, 1995). Figure 4.6 shows the interaction of amantadine with two types of antibiotics that show antimalarial activity: the protein synthesis inhibitor, tetracycline (fig 4.6.a) and the DNA gyrase inhibitor, ciprofloxacin (fig 4.6.b). The antibiotics are slow acting (Yeo and Rieckmann, 1994), therefore the plates were incubated for 96 hours rather than the normal 48 hours. The interaction with tetracycline was synergistic and the interaction with ciprofloxacin showed an exceptional antagonism.

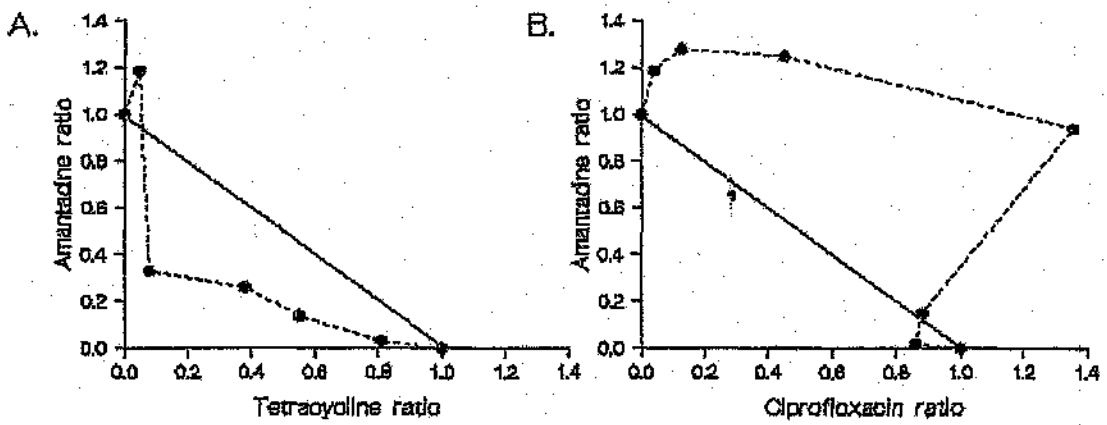


Figure 4.6: Isobologram depicting the interaction of amantadine with antibiotics. A. Tetracyline B. Ciprofloxacin against the chloroquine-resistant (●) strain of *P.falciparum in vitro*

Heparin is a very potent inhibitor of merozoite invasion of red blood cells *in vitro*. The high risk of bleeding associated with therapeutic use of heparin and ambiguous results following treatment have limited its clinical usefulness. There has been a renewed interest in the use of polysaccharides as antimalarials with the development of less

toxic compounds (Kulane *et al.*, 1992). One such substance, currently under investigation is curdlan sulfate (Havlik *et al.*, 1994). Curdlan blocks merozoite invasion, consequently it only exhibits antimalarial activity in the second cycle of the hypoxanthine assay, to overcome this the plates were incubated for 96 hours. The interaction of amantadine with curdlan sulfate was zero-interactive against the chloroquine-resistant strain - see figure 4.7.

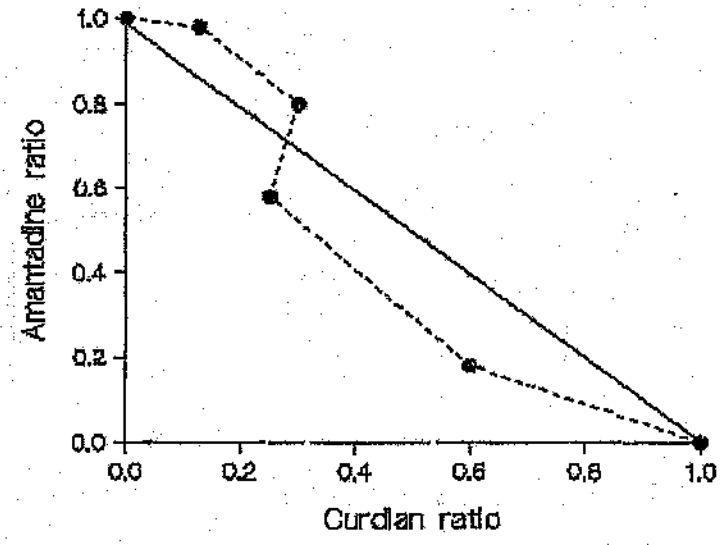


Figure 4.7: Isobologram depicting the interaction of amantadine with curdlan sulfate against the chloroquine-resistant (●) strain of *P.falciparum in vitro*

The results of the combinations of amantadine with both standard and the experimental antimalarials are summarised in table 4.2. The only detrimental drug interaction is that between amantadine and ciprofloxacin. The remaining interactions are either synergistic or additive.

Table 4.2: Summary of interactions of amantadine with other antimalarial drugs against *P.falciparum in vitro*

CLASS	DRUG	INTERACTION	
		CHLOROQUIN E-RESISTANT	CHLOROQUIN E-SENSITIVE
QUINOLINES	Chloroquine	Synergy	Synergy
	Quinine	Synergy	Weak synergy
	Mefloquine	Synergy	----------
	Halofantine	Synergy	----------
ANTI-FOLATES	Pyrimethamine	----------	----------
8-AMINOQUINOLINE	Primaquine	Synergy	----------
ANTIBIOTIC	Tetraycline	Synergy	ND
	Ciprofloxacin	Antagonistic	ND
POLYSACCHARIDE	Curdlan sulfate	Additive	ND

ND = not determined
----- = additive/ zero interaction

In addition to examining the interactions between amantadine and antimalarials, we also examined the effect of amantadine in combination with other chemicals to try to understand the mechanism of action more clearly. The isobolograms of interaction of amantadine with Brefeldin A (a substance which inhibits protein secretion) and leupeptin (a protease inhibitor) are shown in figure 4.8 and 4.9. The interaction with brefeldin A was antagonistic while the interaction with leupeptin was additive.

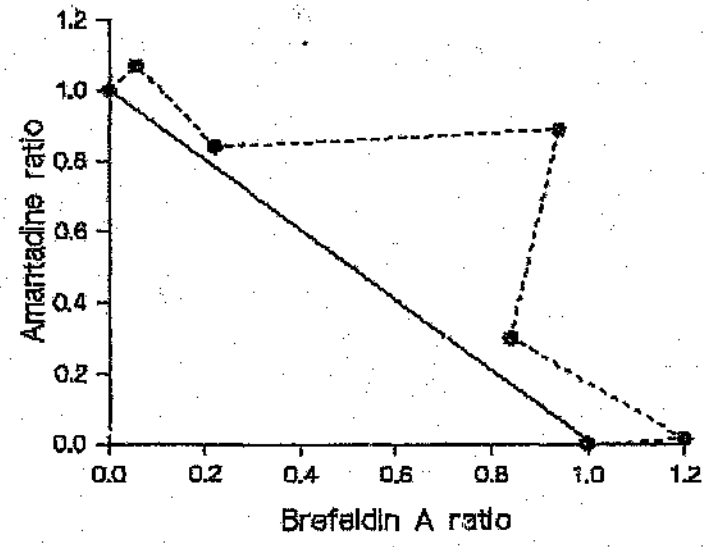


Figure 4.8: Isobologram depicting the interaction of amantadine with brefeldin A (a protein secretion inhibitor) against the chloroquine-resistant (●) strain of *P.falciparum in vitro*

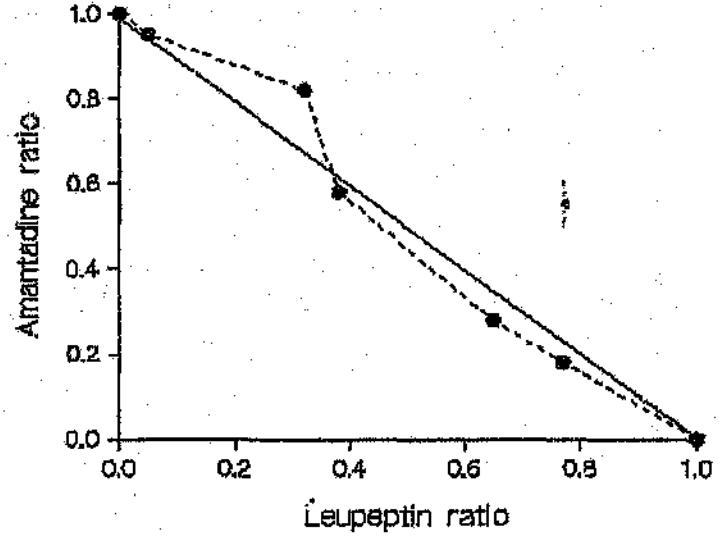


Figure 4.9: Isobologram depicting the interaction of amantadine with leupeptin (a protease inhibitor) against the chloroquine-resistant (●) strain of *P.falciparum in vitro*

4.4 DISCUSSION

An ideal drug combination should be synergistic against the chloroquine-resistant strains, have matching pharmacokinetics and be free of significant side effects and be

relatively inexpensive. Table 4.2 shows that the use of amantadine in combination with other currently employed antimalarial compounds should have no detrimental effect on their antimalarial activity, and may possibly enhance it, particularly against chloroquine-resistant strains. However, the short plasma half life of amantadine is likely to complicate its potential use as a prophylactic agent since the majority of agents have very long half lives.

In addition to providing information about possible adverse drug interactions between antimalarial agents, combination studies can illuminate some aspects of the mechanism of action of drugs. In the rest of the discussion we will critically examine the drug interactions of amantadine from a mechanistic view point.

4.4.1 QUINOLINES

Two distinct patterns emerged from the analysis of combinations of amantadine with the quinoline-based antimalarials - see figure 4.3. The first, is the combination of amantadine with chloroquine - this interaction is synergistic irrespective of chloroquine-sensitivity. The second, is the combination with the other quinolines: quinine, mefloquine and halofantrine - this interaction exhibited synergy with the chloroquine-resistant strain but a zero-interaction or very weakly synergistic reaction in the chloroquine-sensitive strain. This difference probably reflects the presence of two distinct drug-resistance phenotypes (Peters and Robinson, 1991): the chloroquine-resistance phenotype and the mefloquine-resistance phenotype (mefloquine-resistance

is associated with halofantrine-resistance (Rojas-Rivero *et al.*, 1992, Cowman *et al.*, 1994, Peel *et al.*, 1994) and quinine-resistance (Cowman *et al.*, 1994)).

The mode of action of the quinolines is targeted to the food vacuole (Zarchin *et al.*, 1986). However, the degree to which the proposed mechanism of action of chloroquine applies to the other quinolines remains uncertain. There are clearly a number of differences, highlighted by the inherent sensitivity of chloroquine-resistant parasites to mefloquine. For example, the drug efflux as seen in the chloroquine-resistant parasites (Krogstad *et al.*, 1987) has not yet been shown with the other quinolines and the IC_{50} for mefloquine and halofantrine can be decreased by penfluridol, but not by agents that modulate chloroquine-resistance (Peters and Robinson, 1991).

The observed synergy is surprising since all the compounds are weak bases and are expected to concentrate in the food vacuole via proton trapping (De Duve *et al.*, 1974). The resulting alkalinization is believed to have little to do with the antimalarial effect (Ginsburg *et al.*, 1989). Alkalinization is expected to interfere with the uptake of other weak bases producing antagonsitic responses as seen in combinations of chloroquine with ammonium chloride (a prototypical weak base) (Yayon *et al.*, 1985) and chloroquine with quinine/mefloquine/amodiaquin (Stahel *et al.*, 1988). The

synergy is probably connected to the altered transport phenomenon associated with chloroquine-resistance.

4.4.2 8-AMINOQUINOLINE

8-Aminoquinolines are primarily used against gametocytes and hypnozoites (liver stages). Despite its clinical use, little is known about the mechanism by which primaquine is effective, redox-cycling with the generation of active oxygen species is thought to be involved (Augusto *et al.*, 1986). The interaction of primaquine with amantadine follows the pattern seen with other quinolines: mefloquine, quinine and halofantrine. Amantadine is not known to form radicals therefore the synergy observed in the chloroquine-resistant strain is probably due to altered drug transport.

4.4.3 ANTI-FOLATES

Malaria parasites lack the pathways necessary to utilise exogenous pyrimidine, consequently they are dependent on the production of tetrahydrate cofactors for the synthesis of pyrimidine. Compounds such as pyrimethamine, mimic dihydrofolate, thereby blocking the enzyme dihydrofolate reductase, which mediates the final step in tetrahydrofolate synthesis, resulting in parasite death (Ferone, 1977). The combination of amantadine with pyrimethamine shows a zero-interaction response irrespective of chloroquine sensitivity - see figure 4.5. This is the predicted outcome since there is no evidence to suggest that amantadine interferes with any of the enzymes involved in folate metabolism.

4.4.4 ANTIBIOTICS

4.4.4.a Tetracycline

The tetracyclines are representative of a large group of antibiotics that inhibit protein synthesis. The tetracycline family of antibiotics blocks protein synthesis on prokaryotic (bacterial) ribosomes. Their antimalarial activity is believed to be due to inhibition of mitochondrial protein synthesis in the parasite, since mitochondrial ribosomes are similar to prokaryotic ribosomes (Geary and Jensen, 1983, Kiafuengfoo *et al.*,1989, Prapunwattana *et al.*, 1989).

4.4.4.b Ciprofloxacin

The quinolones act against susceptible bacteria by interfering with the activity of DNA gyrase (topoisomerase II). Topoisomerase I and II have been isolated from rodent malaria, but the activity of the isolated parasite enzyme is not diminished by clinically achievable concentrations of drugs that inhibit DNA gyrase activity (Riou *et al.*, 1986). The antiplasmodial action of quinolones seems to be the result of an entirely different, and as yet undefined mechanism. In view of the substantial antagonsitic reaction with amantadine, further investigation of the mechanism of antiplasmodial activity of the fluoroquinolones is essential.

4.4.5 SULFATED POLYSACCHARIDES

Curdlan sulfate inhibits merozoite invasion (personal communication). This arrest is presumably as a result of the polysaccharide coating both merozoites and erythrocytes, thereby forming a mechanical barrier that interferes with membrane fusion. The

antiviral effect of tromantadine, has been attributed to blocking of membrane fusion (Ickes *et al.*, 1990). However, combinations of amantadine and curdlan are additive, inferring the antimalarial effect of amantadine is not due to interference with membrane fusion during merozoite invasion.

4.4.6 BIOCHEMICAL TOOLS

4.4.6.a Brefeldin A

Plasmodial proteins exported to the erythrocyte cytoplasm and membrane mediate nutrient uptake, ionic balance and cytoadherence and are critical for parasite survival (Ansorge *et al.*, 1994). The fungal metabolite, brefeldin A, has become a valuable tool for studying the secretory pathways of various organisms. It has been shown to reorganise the Golgi and block secretory export to the post-Golgi compartments without affecting cellular ATP levels or rates of protein synthesis. Its action is thought to be directed at a G protein associated with or close to the Golgi.

Amantadine is expected to accumulate in the Golgi apparatus due to the molecule being a weak base (Posner *et al.*, 1982). Additionally, part of the antiviral activity is due to the block of the M_2 protein in the Golgi apparatus. The interaction between amantadine and brefeldin A is antagonistic - see figure 4.8. Although, no definite conclusion can be drawn from these results, the stage specific effects of amantadine and brefeldin A are similar (chapter 5). These results warrant further investigation of the effects of amantadine on protein secretion.

4.4.6.b Leupeptin

Intraerythrocytic malaria parasites degrade host erythrocytic haemoglobin as a principal source of free amino acids for parasite protein synthesis. This process involves transport of erythrocytic cytoplasm to the acidic malarial food vacuole, where the heme component of haemoglobin is hydrolysed to free amino acids, which are subsequently incorporated in parasite proteins. The proteolysis of globin by malaria parasites has been implicated in the antimalarial activity of quinolines and appears to involve both cysteine and aspartic proteinase activities (Goldberg *et al.*, 1992). Leupeptin is a cysteine protease inhibitor that inhibits parasite feeding by blocking haemoglobin digestion (Rosenthal, 1995). In the case of chloroquine and mefloquine the reaction of leupeptin is antagonistic confirming the antimalarial effect is as a result of their interaction with a product of host cell digestion (Krugliak and Ginsburg, 1991). The combination of amantadine with leupeptin is additive inferring that the antimalarial effect is not due to inhibition of cyteine protease activity in the food vacuole. However, amantadine could still interfere with the parasite feeding process by some other mechanism.

4.5 SUMMARY

In conclusion, the mechanism of action of amantadine appears to be distinct from other known antimalarials. The observed synergy with quinolines probably involves changes in the transport of these drugs in the chloroquine-resistant phenotype.

CHAPTER FIVE - PARASITE DEVELOPMENT

5.1 INTRODUCTION

Following merozoite invasion of the RBC the intraerythrocytic development is divided into three morphologically distinct stages (see figure 5.1):

a) the rings (0-24 hrs post infection)

b) the trophozoites (24-36 hrs post infection)

c) the schizonts (36-48 hrs post infection)



Figure 5.1: Schematic representation of intraerythrocytic cycle of *P.falciparum*

Information on the stage selective effects of amantadine could provide important clues to the mechanism(s) of action of the drug and the establishment of a treatment regimen.

The effect of amantadine on the development of each stage will be examined, the speed of onset of antimalarial activity against ring and troph stages and the morphological changes as determined by light microscopy will be investigated.

5.2 EXPERIMENTAL PROCEDURE

5.2.1 STAGE DEPENDENCE OF DRUG EFFECTS

5.2.1.a Morphological effects on development through the life cycle (Zhang *et al.*, 1986)

A highly synchronized young ring culture (2% parasitemia and 5% haematocrit) was aliquoted into 2.5 cm glass plates (time = 0 hrs). The plates were treated as described below and incubated in a candle jar at 37°C for 72 hrs:

Group 1: Control group, parasites were cultured in drug free medium throughout

Group 2: Parasites were exposed to drug from the ring stage (i.e. 0 hrs) onwards

Group 3: Parasites were exposed to drug from the trophozoite stage (i.e. 24 hrs) onwards

Group 4: Parasites were exposed to drug from the schizont stage (i.e. 32 hrs) onwards

The drug concentration selected was sufficient to produce >90 % reduction in ^{3}[H]-hypoxanthine incorporation (amantadine = 50 μM). Thin blood smears were made at 8 hour intervals, stained with Giemsa stain and examined under a light microscope. The parasitemia and stage distribution of parasites was determined.

5.2.1.b The effect of amantadine on merozoite invasion (Ward *et al.*, 1993)

Erythrocytes harbouring parasites at late schizont stages (i.e. majority of schizonts contained 16 nuclei) were suspended in medium (haematocrit = 10 %) and passed 10 times through a 25 G needle to release the merozoites. Unsheared parasites and uninfected erythrocytes were pelleted twice (60s, 1100 g) and the pellet was discarded. The supernatant was spun for 90s at 1900 g to pellet the merozoites. The merozoites were washed twice in medium and resuspended in normal medium. The merozoite suspension was mixed with uninfected red blood cells treated with 50 μM amantadine or untreated. Parasites were incubated at 37°C in a candle jar for 8 hours. Thin smears were made and stained with Giemsa stain then the parasitemia was determined.

5.2.2 SPEED OF ONSET OF ANTIMALARIAL ACTIVITY

5.2.2.a Time dependence (Krugliak and Schlesinger, 1991)

Synchronized cultures at the ring and trophozoite stage respectively (1% parasitemia and 1% haematocrit) were aliquoted and treated with different concentrations of amantadine. At various time intervals (1,2,4 and 8 hrs), duplicate samples were removed. Drug was washed out of parasites by three wash cycles of centrifugation in

drug free medium. After centrifugation the cell pellet was suspended in complete hypoxanthine free medium. Aliquots were distributed to each of three wells in a 96-well microtitre plate. At 8 hrs ^{3}[H]-hypoxanthine was added, plates were harvested 48 hours later and the radioactivity counted. IC_{50} values were determined.

5.2.2.b Second cycle studies

Young ring forms (<12 hrs) have relatively little metabolic and synthetic activity. Consequently, drug effects were also determined in the subsequent (i.e. second) cycle using a reinvasion assay.

Drug sensitivity over two cycles was determined using a modification of the hypoxanthine technique. Plates were prepared as previously described but radiolabelled hypoxanthine was added at 66 hours and the plates were harvested 18 hours later (84 hrs after start).

5.2.3 THE EFFECT OF AMANTADINE ON PROTEIN SYNTHESIS

The incorporation of ^{3}[H]-isoleucine, an amino acid that is relatively scarce in haemoglobin, serves as a convenient marker for cystolic protein synthesis by the intraerythrocytic stages of the parasite (Blum *et al.*, 1984). The effect of amantadine on ^{3}[H]-isoleucine incorporation was assessed essentially as described for parasite growth.

5.2.3.a Preparation of parasites

A sample of routinely synchronised stock cultures (mid-troph stage) was diluted in medium containing uninfected erythrocytes to yield a final haematocrit of 2 % and parasitemia of 2 %. Parasite suspension was aliquoted into 96 well plates, pre-treated with different concentrations of amantadine.

5.2.3.b Labelling parasites

^{3}[H]-isoleucine (1 μCi/ml) was added (time = 0 hrs). Parasites were incubated in a candle jar at 37°C.

5.2.3.c Harvesting parasites

Plates were harvested at 2, 4, 6 and 8 hours and filters were processed for scintillation counting as previously described.

5.2.3.d Data analysis

Counts were corrected for incorporation of isoleucine into uninfected cells.

5.3 RESULTS

5.3.1 STAGE DEPENDENCE OF DRUG EFFECTS

The distribution of forms in a synchronous control culture are shown as open points (fig 5.2, 5.5, 5.7). Maturation of the ring forms to trophozoites occurs in about 20 - 24 hours. Schizonts are present between 36 - 42 hours. Reinvasion is observed at 40 - 48 hours and is completed at 56 hours.

5.3.1.a Rings

When ring stage parasites were treated with amantadine (50 μM), the effect of amantadine treatment is seen within 8 hours. The maturation of the ring stages is inhibited. Few trophs are formed and no schizonts develop (fig 5.2), At the light microscopic level the morphology of the treated ring forms was indistinguishable from that of untreated rings (fig 5.3). The IC_{50} for the arrest in ring development was 20.47 ± 5.69 μM. (fig 5.4). The inhibition of ring development to trophs was largely unaffected at the IC_{50} concentration (5 μM), making it probably of little clinical significance.

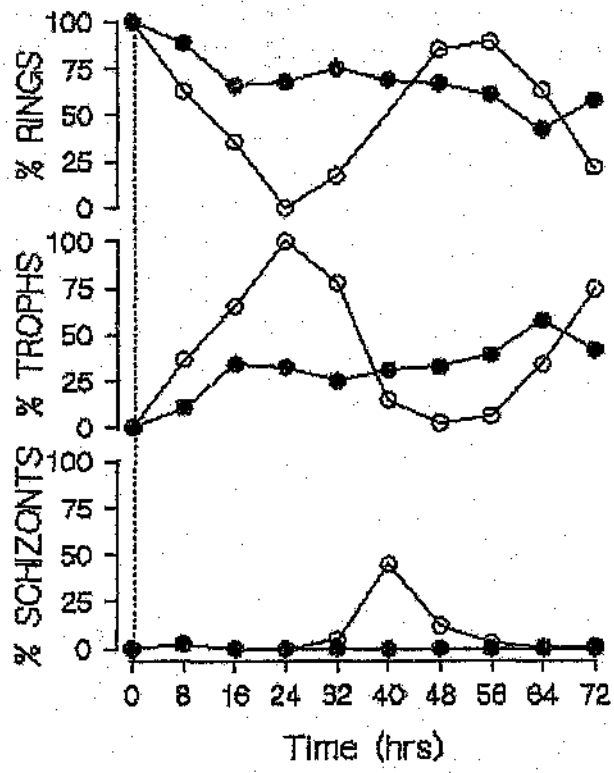


Figure 5.2: Relative stage distribution in chloroquine-resistant parasites treated with amantadine (50 μM) at ring stage (0 hrs) (●) and untreated control (○)

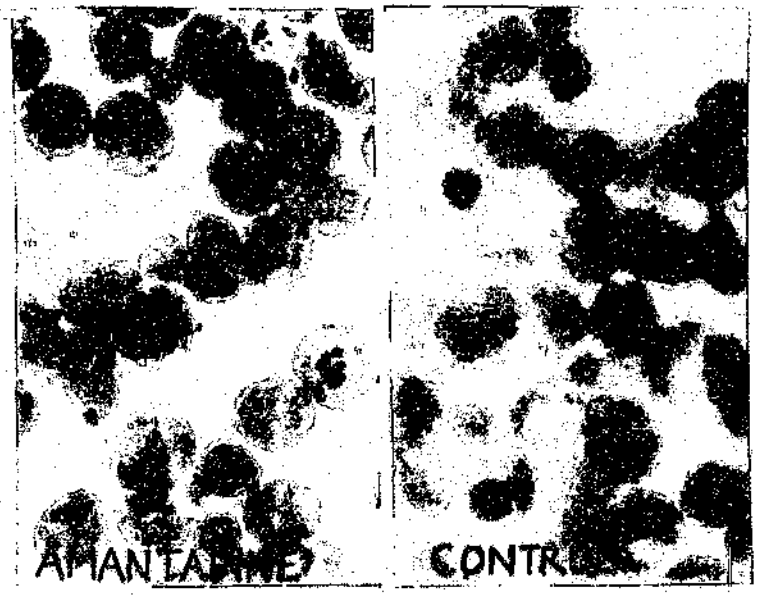


Figure 5.3: Morphological changes occuring after 24 hrs in chloroquine-resistant parasites treated with amantadine (50 μM) at the ring stage (0 hrs) compared with untreated control

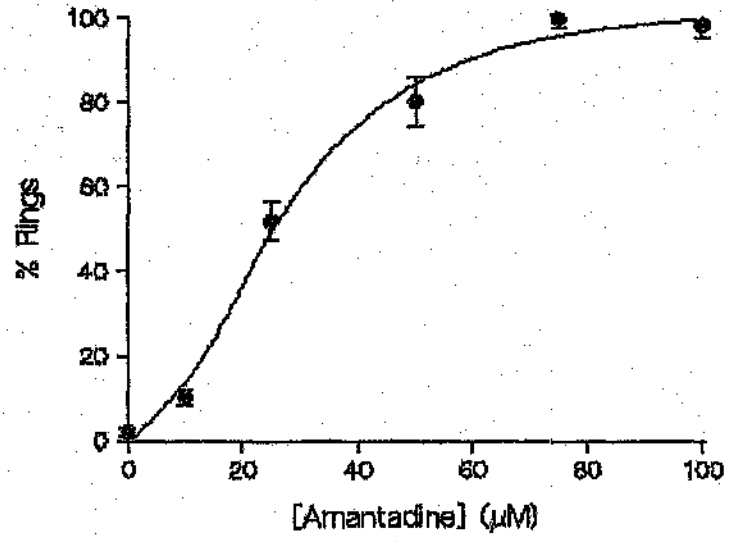


Figure 5.4: Dose response curve of ring block 24 hours after treatment with amantadine. Corresponding untreated parasites were 100 % trophs

5.3.1.b Trophs

When amantadine was added when the majority of the parasites were trophozoite forms (24 hours), the effect on the stage distribution was visible within 8 hours (fig 5.5). Maturation of the trophs was retarded and parasites were morphologically abnormal. No schizonts formed. Light microscopic examination of the Giemsa-stained

smears revealed morphological changes within 2 hours after drug administration. Initially, the appearance of a large food vacuole, seldom seen in untreated parasites, was noted. Within the large food vacuoles, pigment granules were often clumped and occupied the internal space of the vacuole. There was no evidence of undigested globin accumulating within the food vacuole. The degree of food vacuole swelling tended to increase with prolonged exposure time to amantadine. Eventually the vacuole occupied up to 60-70 % of the parasite volume. Continued exposure resulted in a loss of structural integrity and disintegration resulting in empty vacuoles remaining within the host cell membrane. The parasite cytoplasm becomes more dense and by 16 - 24 hrs the parasites appeared to disintegrate and disappear completely.

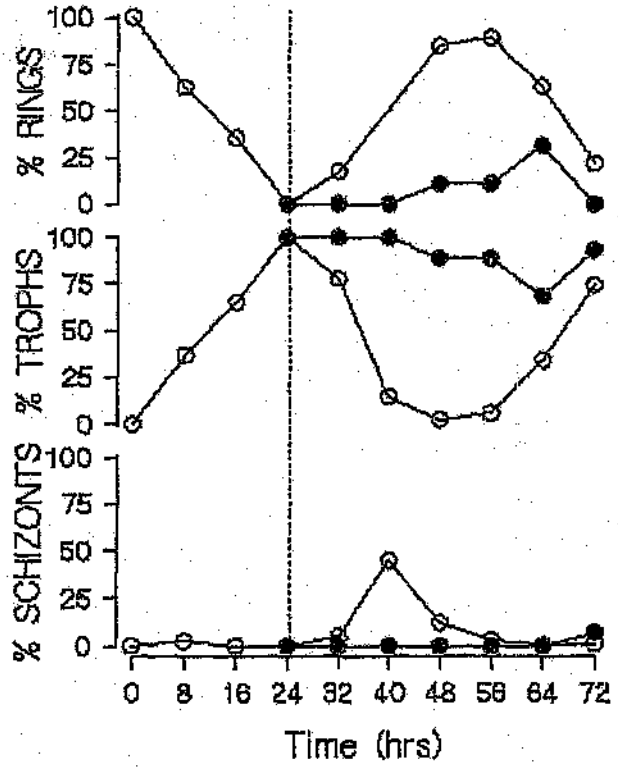


Figure 5.5: Relative stage distribution in chloroquine-resistant parasites treated with amantadine (50 μM) at troph stage (24 hrs) (●) and untreated control (○)

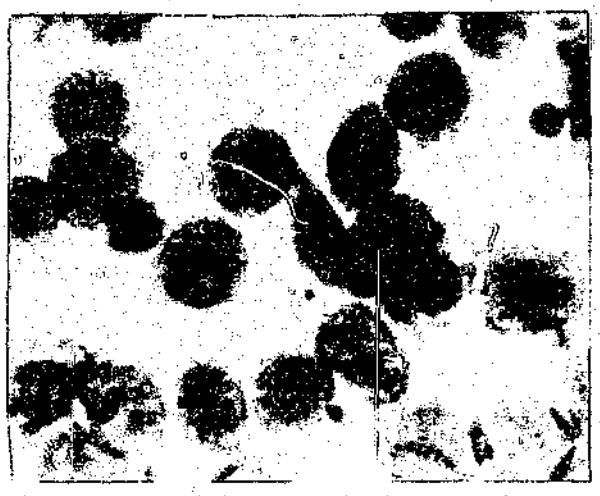


Figure 5.6: Morphological changes occuring after 8 hrs in chloroquine-resistant parasites treated with amantadine (50 μM) at the troph stage (24 hrs)

5.3.1.c Schizonts-merozoites

The effect of exposure of late trophozoite - early schizont stages to amantadine is shown in figure 5.7. Sensitivity to amantadine decreased as nuclear division proceeded through shizogony to segmentation. Once segmenters were formed they were essentially unaffected by the drug. Schizonts released merozoites, although with a slight delay. The merozoites formed were morphologically identical to those in control cultures. Reinvasion of the erythrocytes occurred, as evidenced by the appearance of new ring stages.

Isolated *P.falciparum* merozoites exposed to amantadine, invaded and developed in erythrocytes in a manner similar to that observed in the controls (fig 5.8) - the observed difference was not significant. These results confirm that reduction in new

invasions due to treatment with amantadine is not due to a failure of released merozoites to invade erythrocytes but rather a failure of schizonts to mature.

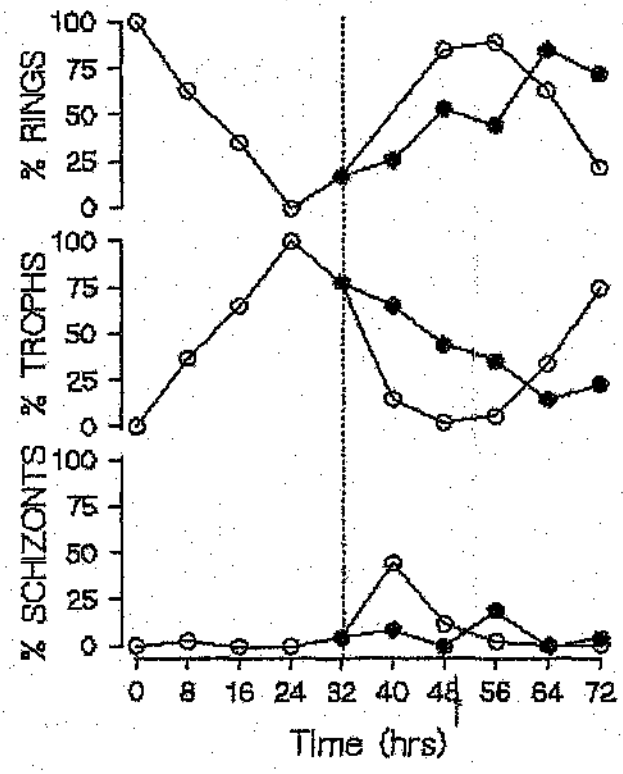


Figure 5.7: Relatvie stage distribution in chloroquine-resistant parasites treated with amantadine (50 μM) at schizont stage (32 hrs) (●) and untreated control (○)

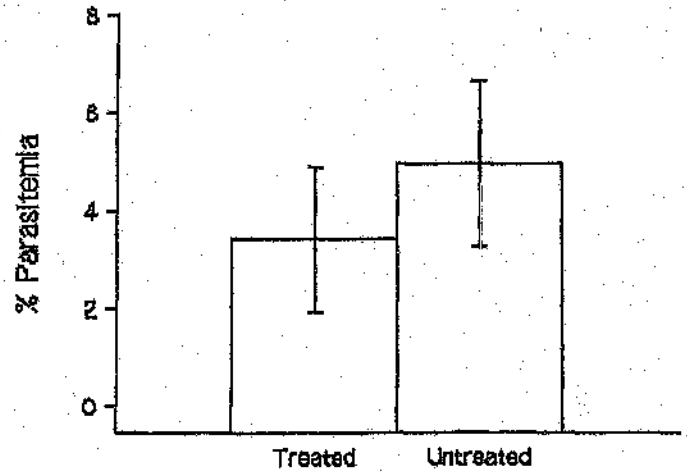


Figure 5.8 : Parasitemia following exposure of isolated *P.falciparum* merozoites to erythrocytes suspended in normal or amantadine (50 μM) containing medium

5.3.1.d Time course of stage effects

When the ring stage parasites were treated, the parasitemia remained the same despite the delayed development (fig 5.9), however, with continued exposure the culture inevitably disappears due to the disruption of the cycle (fig 5.10). When trophozoite and schizont forms are exposed to amantadine, the parasitemia decreases significantly (fig 5.9) due to parasite death and failure to form schizonts and thus reinvade. The schizont stage is the least sensitive to the effects of amantadine, showing an increase in parasitemia (fig 5.10). The absence of an effect on the formed schizont is probably greater than illustrated since even highly synchronous cultures exhibit at least a 4 hour window in ages.

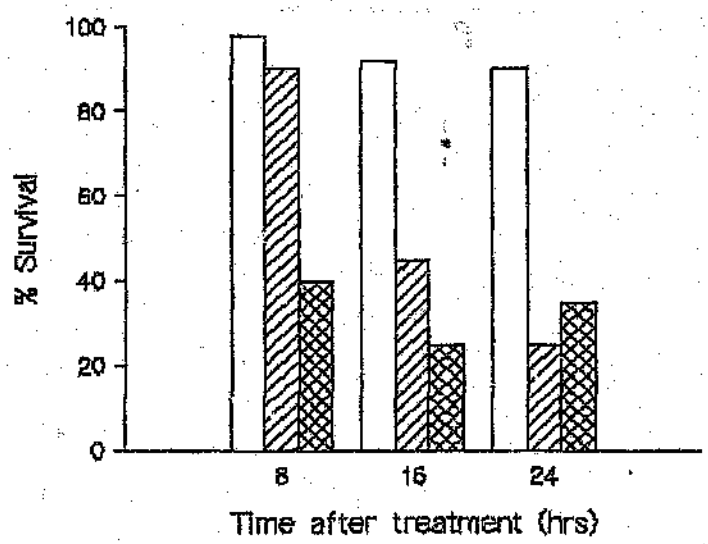


Figure 5.9: Relative parasite survival after amantadine treatment at different stages (□) rings , (/) trophs and (X) schizonts

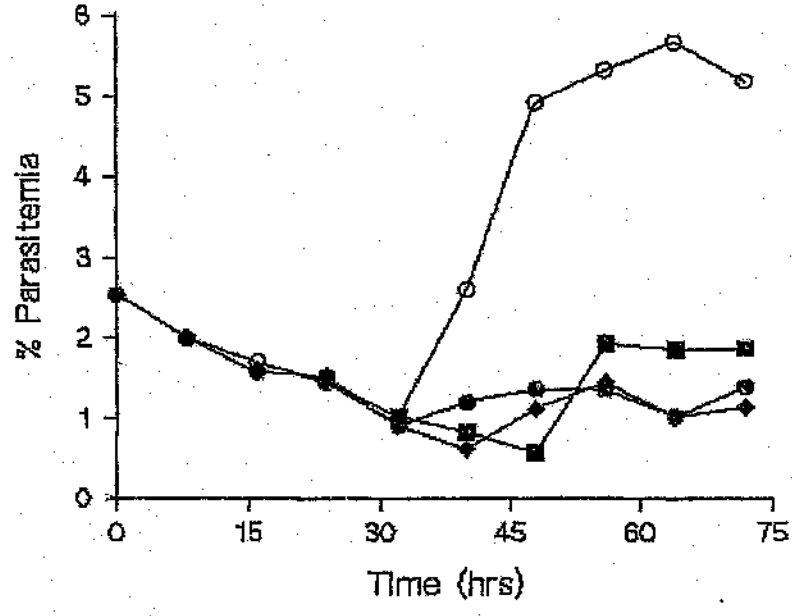


Figure 5.10: Total parasitemia after amantadine treatment at different stages (○) control, (●) rings, (◆) trophs and (■) schizonts

5.3.2 SPEED OF ONSET OF ANTIMALARIAL ACTIVITY

The developmental arrest of the ring stage parasites by amantadine is reversible and does not affect the capability of previously arrested cells to progress to the next generation (fig 5.11). The effect of amantadine on the ring stage parasites indicates that the drug has no long-term and irreversibly toxic effect on this developmental stage. When trophozoites were cultured in the presence of amantadine for more than 4 hours, an increasing number of morphologically abnormal parasites were observed, and the rate of reinvasion decreased accordingly (fig 5.11). The time dependent effects of amantadine contrast with those seen with chloroquine. The effect of chloroquine was directly proportional to the time of exposure for both ring and the trophozoite

stages (figure 5.12). The ring stage parasites were less susceptible to chloroquine than the trophozoites. Exposure of the trophs for 8 hrs was equal to that seen in continuous exposure. The effect of chloroquine on both ring and trophs stages was irreversible.

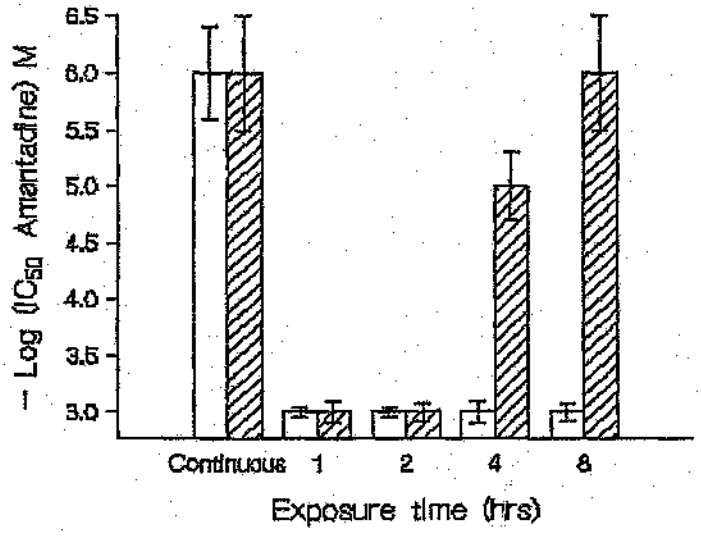


Figure 5.11: Time dependence of response of parasite to amantadine at different stages, rings (open bars) and trophozoites (stripped bars). Results are expressed as - log IC_{50} i.e. higher values correspond to greater drug effect

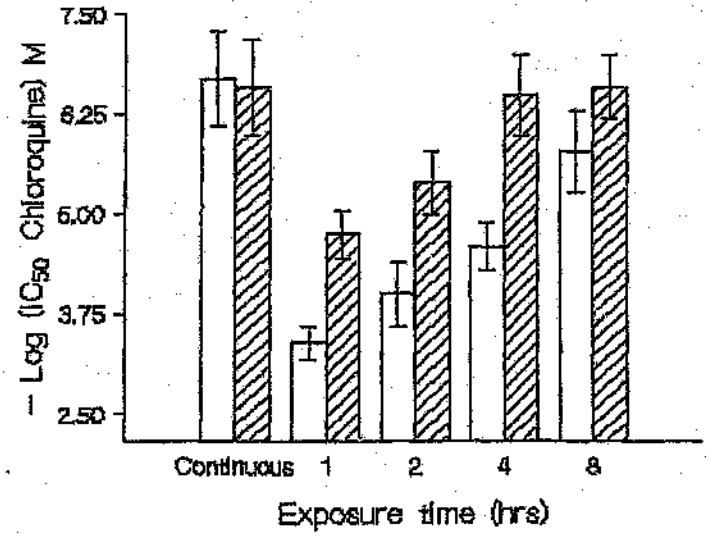


Figure 5.12: Time dependence of response of parasite to chloroquine at different stages, rings (open bars) and trophozoites (stripped bars). Results are expressed as - log IC_{50} i.e. higher values correspond to greater drug effect

In view of the relatively slow onset of action against the trophozoite stage, we determined the IC_{50} of amantadine of a second cycle. Fig 5.13 compares the single and double cycle IC_{50} of amantadine in the FCR-3 (chloroquine-re[illegible] strain of *P.falciparum*. Prolonged exposure reduces the IC_{50} almost 10 fold to below 1 μM.

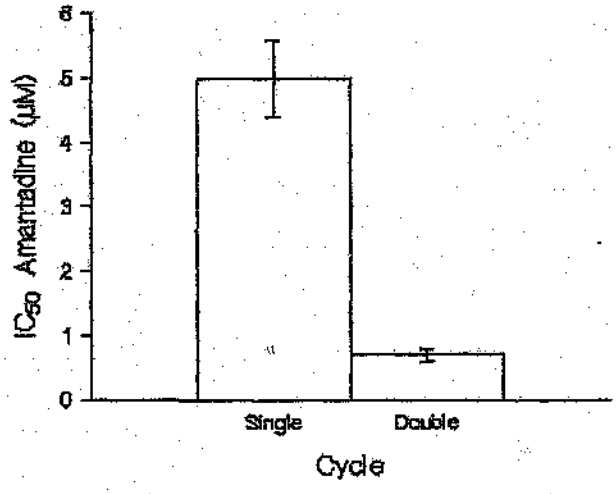


Figure 5.13: Comparison of single and double cycle IC_{50} values for amantadine in the chloroquine-resistant (FCR-3) strain of *P.falciparum in vitro*

5.3.3 THE EFFECT OF AMANTADINE ON PROTEIN SYNTHESIS

Isoleucine is incorporated into all stages of parasite growth. The greatest incorporation occurs during the mature stages of parasite growth (results not shown). Figure 5.14 shows the accumulation of isoleucine and thus protein synthesis in trophozoites monitored over a period of 8 hours compared to uninfected red blood cells.

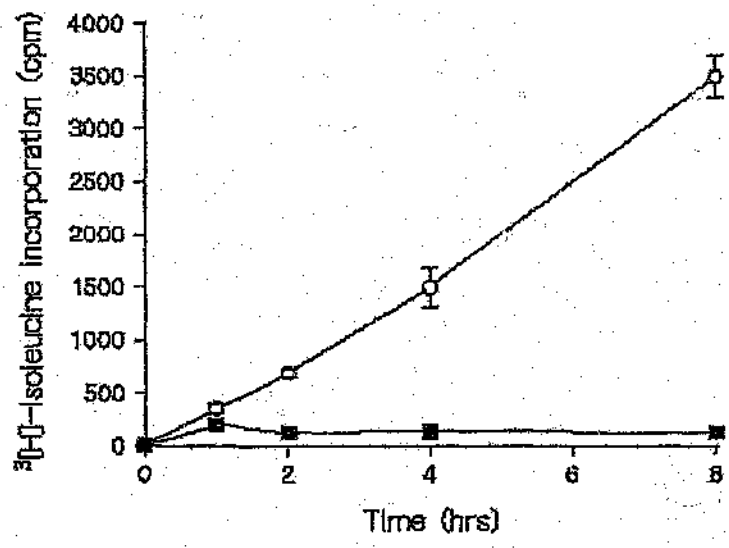


Figure 5.14: Time course of protein synthesis by trophozoite infected (○) and uninfected (■) red blood cells

The effect of amantadine on isoleucine uptake in the chloroquine-resistant (FCR-3) strain of *P.falciparum* is shown in figure 5.15. Protein synthesis in the trophozoite stage was only significantly inhibited following exposure to amantadine for 8 hours. The same concentrations of amantadine produce irreversible lethal effects in the majority of parasites, after 4 hours of exposure. The inhibition observed following treatment for 8 hours most likely reflects non-specific toxicity as a result of parasite death. The antimalarial effect of amantadine is not due to the block of protein synthesis.

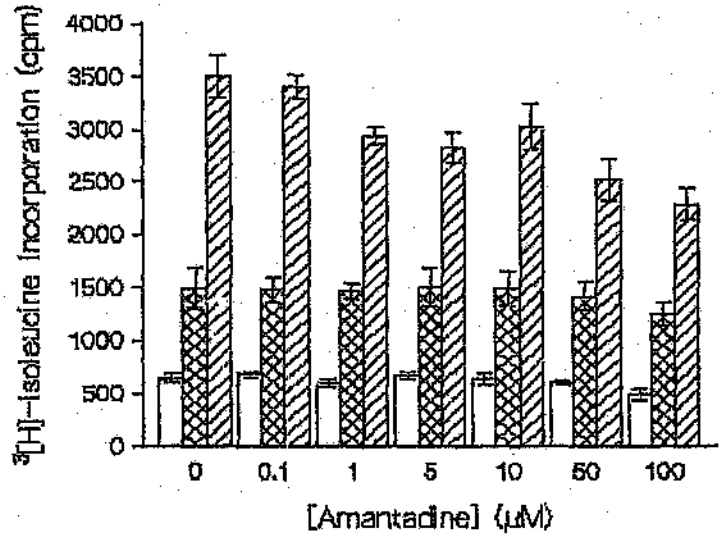


Figure 5.15: Isoleucine uptake (expressed as cpm/sample) following exposure to amantadine for 2 hrs (□), 4 hrs (X) and 8hrs (/)

5.4 DISCUSSION

5.4.1 MECHANISM OF ACTION

Parasite feeding is probably not the target of amantadine. Despite changes in the morphology of the food vacuole there is no evidence haemoglobin digestion has been interrupted. Undigested haemoglobin does not accumulate in the food vacuole as seen with the cysteine protease inhibitors and hemazoin continues to be present. In addition, stage sensitivity data does not support haemoglobin digestion as a target: active haemoglobin digestion is restricted to the late ring to late trophozoite forms. The enlargement of the food vacuole probably reflects osmotic swelling due to the accumulation of high concentrations of drug in the vacuole, by virtue of its lysosomotropic character (Krogstad and Schlesinger, 1987). Vacuolarization in other cell types following treatment with amantadine has been documented (Pita and Perez, 1977).

The molecular target of amantadine in the ring and trophozoite stages is expected to be identical. The observed differences in sensitivity for the ring and trophozoite stages may reflect the differences in the consequence of action. The effects are reversible for the former over a long period, but lethal in the latter. Various aspects of cellular metabolism including DNA, RNA, protein synthesis, phospholipid turnover and Ca^{2+} metabolism demonstrate stage dependence (Pfaller *et al.*, 1982, Gritzmacher and Reese, 1984, De Rojas and Wasserman, 1985).

Overall, the ring stage is regarded as metabolically sluggish in comparison with the highly active trophozoite form. A significant activity associated with the early ring stage (as early as 6 hours after invasion) is the export of parasite proteins to the host cell membrane and changes in the permeabilization of the host erythrocyte membrane (Kutner *et al.*, 1985). In light of this, it is particularly interesting that the stage-specific effects of amantadine parallel those seen for Brefeldin A and low temperature. Ring stage parasites are arrested in their development but remain viable. When added to trophs, the drug is lethal. Brefeldin A and low temperature inhibit protein secretion and the biogenesis of the plamsa membrane by interfering with the intracellular membrane flow (Thevenod *et al.*, 1994).

5.4.2 TREATMENT REGIMENS

The stage sensitivities of the parasite to drugs has important implications for the antimalarial activity *in vivo*. The majority of deaths occur within 24 hours of admission

- death is usually from the current "brood" of parasites. Sequestration is considered to be the essential pathological process in severe falciparum malaria. Sequestration leads to organ dysfunction and damage as a result of the packing of parasites into the microvasculature of vital organs such as the brain, heart, liver and kidneys.

Sequestration in deep vascular beds begins to occur at the time (roughly 26 hours after schizogony) when pigment is first visible in the new brood of parasitized erythrocytes. If the cycle of parasite development is uninterrupted and there is no clearance, the number of sequestered cells will eventually equal the number that had been circulating hours previously as rings. Drugs acting early in the cycle would prevent maturation to the pathogenic phase, whereas drugs acting at very late stages would not do so. The ideal drug should be active against all stages (White, 1988).

The cytotoxic effect of amantadine is dependent on the stage and length of exposure. Amantadine is active against both ring (cytostatic) and trophozoite stages (cytocidal). However, the effect against the ring stage occurs above concentrations that are likely to occur *in vivo* therefore the time window of activity of amantadine is similar to that found for the quinolines ie late ring to late trophozoite stage (fig 5.16). Amantadine's onset of action is relatively slow, reinforcing the idea that administration of amantadine would need to be in combination with more rapidly acting antimalarials as is seen with antibiotics (Yeo and Rieckmann, 1994). Time of exposure is likely to play a major

role in the outcome of therapy with amantadine, a longer course of treatment (7-10 days) rather than the standard 3-4 days for quinolines should give the best results.

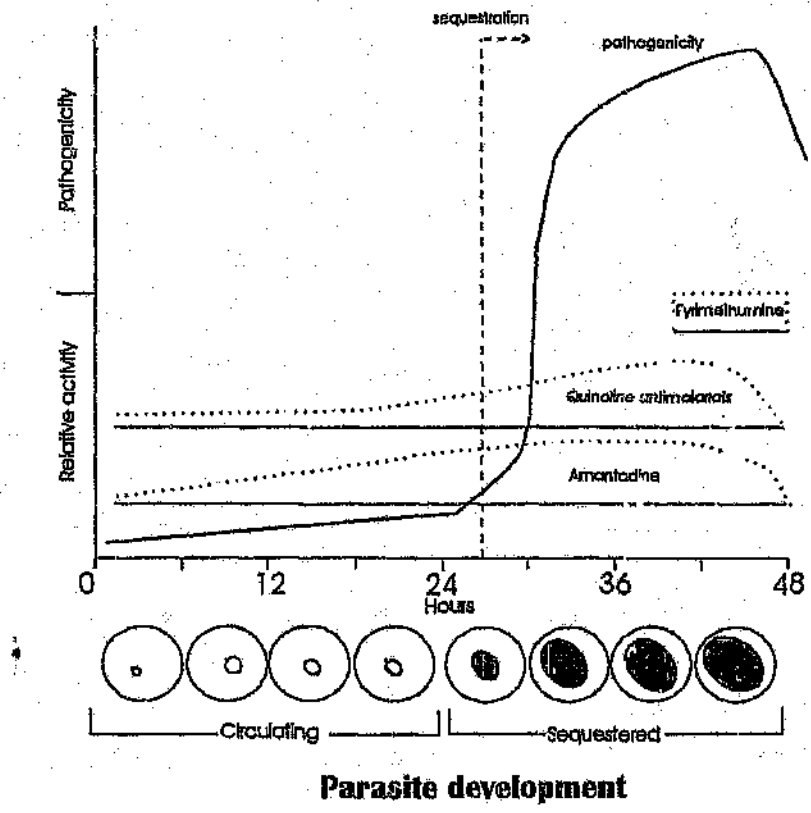


Figure 5.16 : Comparison of time window of effect of amantadine with other antimalarials

5.5 SUMMARY

Amantadine inhibits progression through the erythrocytic cycle, however, the drug has no long-term and irreversible toxic effect on this developmental stage. In contrast, the viability of trophozoites is affected by amantadine within a comparatively short time of treatment.

CHAPTER SIX - pH

6.1 INTRODUCTION

Accumulation of a weak base in an acidic compartment is expected to correlate with the magnitude of the pH gradient. The mechanism of accumulation can be described as follows (De Duve *et al.*, 1974). The drug diffuses from the extracellular medium into the parasite in its free base (unprotonated) form. Once it reaches a intracellular compartment, as this has a lower pH than the extracellular medium, the drug becomes protonated. The protonated drug cannot freely translocate across membranes and thus the drug becomes trapped in the acidic compartment. Most of the drug accumulates in the the acidic food vacuole of the parasite as shown in figure 6.1 (Krogstad *et al.*, 1987).

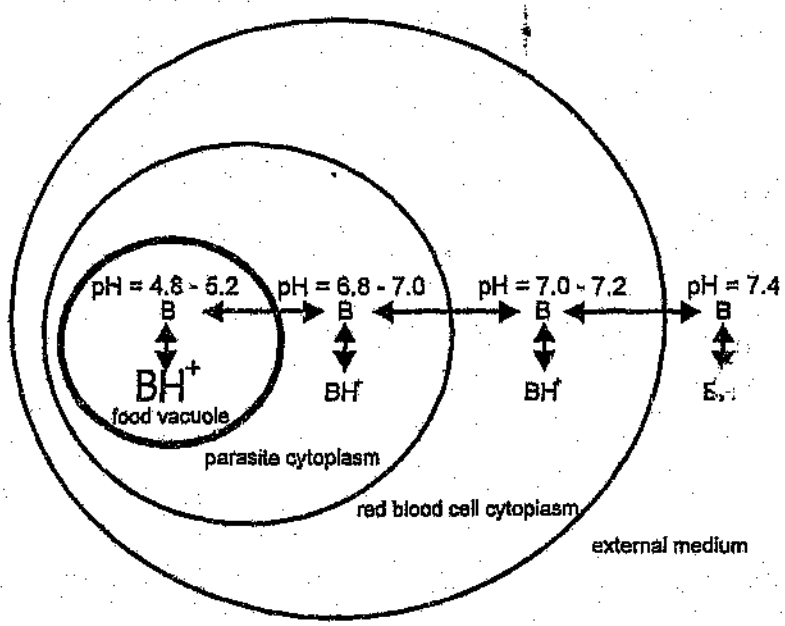


Figure 6.1 : Schematic diagram showing accumulation of a weak base against the pH gradient in *P.falciparum*

This protonation depletes the vacuole of protons and thus raises the pH. Although the vacuolar proton pump provides the organelle with some capacity to buffer these changes, a massive influx of drug would be expected to raise the pH. The proteolytic enzymes of the food vacuole function optimally at an acidic pH (typically pH 5) and show reduced activity as the pH is raised (Slater, 1993).

In this section, the outcome of modulating the pH gradient between cells and the medium on drug sensitivity and the effect of amantadine on vacuolar pH will be investigated.

6.2 EXPERIMENTAL PROCEDURE

6.2.1 THE INFLUENCE OF pH ON DRUG SENSITIVITY

6.2.1.a Modulation of external pH (Ginsburg and Stein, 1991)

Hypoxanthine-free medium, supplemented with 5% sodium bicarbonate, was pH adjusted using HCl/NaOH to give the different starting pH values, the medium was sterilized by filtration through a 0.22 μm filter. Exposure to drug occurred as previously described. Experiments were performed at the same time to prevent inoculum size effects.

6.2.1.b Dissipation of the pH gradient (Krogstad *et al.*, 1985)

Growth of chloroquine-resistant parasite in fixed ratios of amantadine and ammonium chloride was assessed and the results plotted as isobologram.

6.2.1.c Amine group

Drug sensitivity of amantadine was compared to adamantane, a related compound sharing the hydrocarbon cage of amantadine but lacking the amine group.

6.2.2 THE EFFECT OF AMANTADINE ON THE PARASITE'S ACIDIC COMPARTMENT

Acridine orange (fluorescent probe) accumulation is driven by a pH gradient in many types of cells and isolated acidic organelles, including malaria parasites (Ginsburg *et al.*, 1989).

6.2.2.a Preparation of sample

Cultures of parasitized red blood cells which had reached the trophozoite stage were enriched using the gelatin floatation technique. Parasites were washed 3 times in PBS supplemented with 10 mM glucose. Parasites were used immediately.

6.2.2.b Acridine orange accumulation

Acridine orange was added to 3 ml PBS-G (final concentration = 1 μM) and allowed to equilibrate in a polycarbonate cuvette before an aliquot of pRBCs was added (0.8 % haematocrit). Time dependent changes in fluorescence were monitored at 495 nm excitation and 525 nm emmission in a ratio mode. All measurements were performed at room temperature using a Perkin-Elmer LS-50 spectrofluorometer with continuous stirring. Aliquots of drug were added from stock solutions once equilibrium was achieved. The experiment was terminated with the addition of excess NH_4Cl (25 mM) - which released the accumulated dye.

6.2.2.c Data analysis

The relative fluorescence quenching (Q) was determined from the plot of the fluorescence changes over time as shown in figure 6.2. The relative fluorescence quenching is a direct measure of dye uptake since :

$$Q = \frac{[AO]_i}{[AO]_o}$$

where i = food vacuole and o = external medium

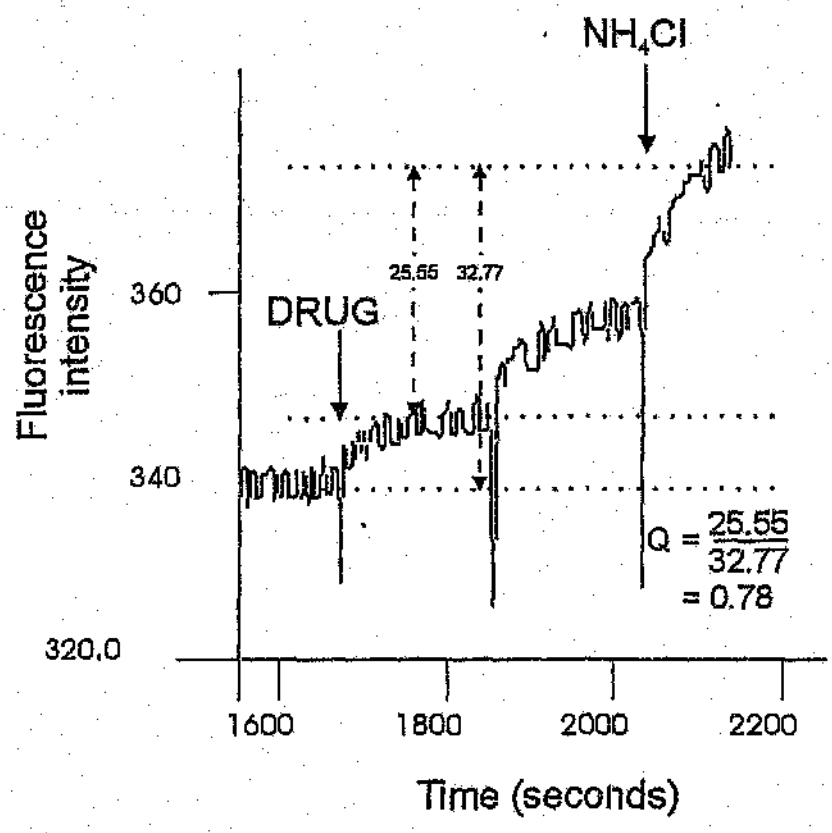


Figure 6.2: Calculation of relative flourescence quenching (Q) from the plot of fluorescence against time

Based on the Henderson-Hasselbalch equation acridine orange accumulation is expected to be governed by the the equation :

$$\frac{[AO]_i}{[AO]_o} = \frac{[H+]_i}{[H+]_o}$$

i.e. $$Q = \frac{[H+]_i}{[H+]_o}$$

therefore $$\log Q = pH_o - pH_i$$

A plot of fluorescence quenching against pH_o results in a straight line (results not shown), however, pH_i cannot by accurately determined due to binding of acridine orange to cellular components. Fluorescence quenching data can be used to calculate changes in pH_i in the prescense and absence of drug using the derived equation :

i.e. $$\log Q - \log Q' = pH_i - pH'_i = dpH_i$$

since the complicating binding factor is cancelled out.

6.2.3 THE EFFECT OF pH ON OSMOTIC FRAGILITY

Morphological studies indicate amantadine accumulates to high concentrations in the food vacuole. Amantadine, like other amphiphiles is expected to partition into the membrane to some critical concentration, depending on the hydrophobic and ionic nature and cause membrane disruption. The effect of amantadine on membrane integrity at pH 5 (corresponding to food vacuole pH) was compared to the effect at physiological pH using a hemolytic assay (Ginsburg and Krugliak, 1988).

6.2.3.a Preparation of sample

Fresh non-parasitized erythrocytes were washed three times in HEPES buffered saline (HBS) (pH 7.4) and resuspended in HBS to give 20 % haematocrit.

6.2.3.b Hemolysis assay

Twenty µl of the erythrocyte suspension was mixed with 1 ml hypotonic HBS at pH 7.4 or pH 5 containing different concentrations of drug. The suspension was incubated at 37 °C for 30 min, then spun down and the hemoglobin absorbance in the supernatant determined spectrophotometrically at 540 nm.

6.3.3.c Data analysis

The absorbance with the drug was compared with the absorbance in the absence of drug. A ratio > 1 was defined as destabilization, a ratio <1 was defined as stabilization.

6.3 RESULTS

6.3.1 INFLUENCE OF pH ON DRUG SENSITIVITY

Incorporation of ^{3}[H]-hypoxathine was unaffected by the range of pH used (results not shown). Dose response curves for amantadine and the antimalarials chloroquine, quinine and mefloquine at different pH values were obtained. Figure 6.3 plots the log IC_{50} values against the external medium pH for chloroquine, the IC_{50} values of chloroquine were logarithmically dependent on the external pH in both chloroquine-resistant (FCR-3) (r = 0.986) and chloroquine-sensitive (3D7a) (r = 0.954) parasites. These results agree with those previously reported (Yayon *et al.*, 1985, Geary *et al.*, 1990) and support the assumption that the pH gradient is principally responsible for

the accumulation of chloroquine to a critical toxic concentration inside the parasite food vaucole.

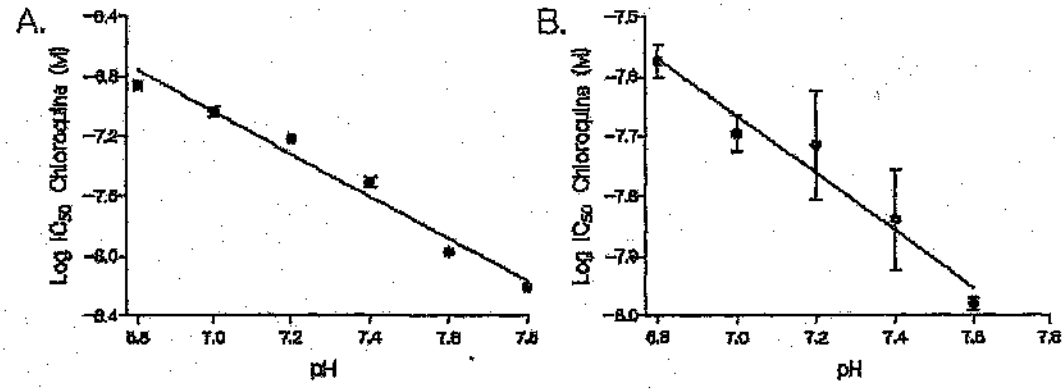


Figure 6.3 : Dependence of IC_{50} of chloroquine on extracellular pH. A. Chloroquine-resistant (FCR-3) B. Chloroquine-sensitivie (3D7a) strain of *P.falciparum*

Amantadine accumulation is expected to be governed by a similar principal. The IC_{50} values are expected to decrease with increasing pH as the concentration of the basic species, which has been demonstrated to cross the membrane (Johnson *et al.*, 1981), increases. The chloroquine-resistant (FCR-3) strain showed a non-linear dependence on external pH. The plot of pH against the log of amantadine concentration gave a parabolic function with a minimum IC_{50} at pH = 7.03 (figure 6.4a). The chloroquine-sensitive (3D7a) strain showed a linear-dependence on the external pH (r = 0.85) (figure 6.4b).

The plot of the log IC_{50} values against the external medium pH for the monobasic antimalarials quinine and mefloquine are shown in figure 6.5. The sensitivity of the

chloroquine-resistant (FCR-3) strain to quinine was found to be logarithmically dependent on pH ($r = 0.9575$) as expected. However, the sensitivity to mefloquine was independent of pH, this probably reflects the fact that mefloquine is tightly bound to the membrane - uptake is believed to be via a electrochemical gradient rather than pH gradient.

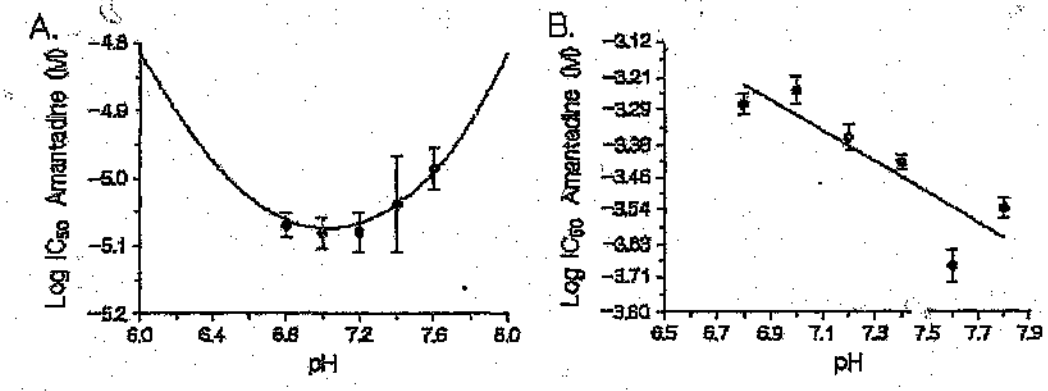


Figure 6.4 : Dependence of IC_{50} of amantadine on extracellular pH. A. Chloroquine-resistant (FCR-3) B. Chloroquine-sensitivie (3D7a) strain of *P.falciparum*

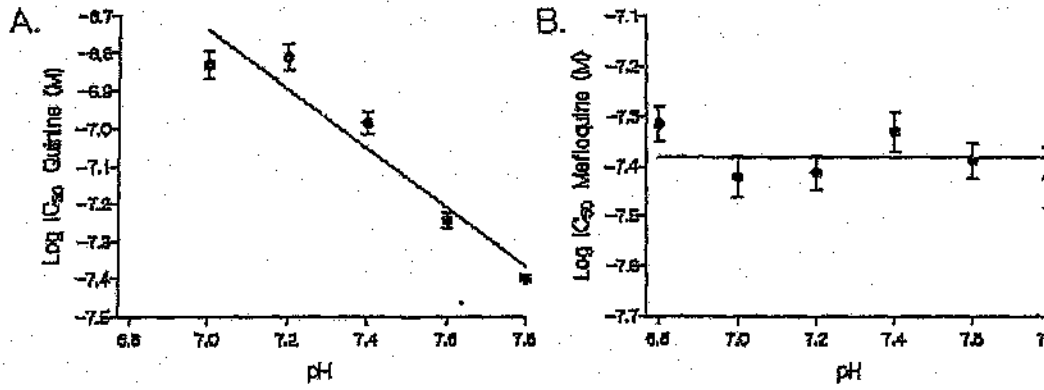


Figure 6.5 : Dependence of IC_{50} of monobasic antimalarials on extracellular pH on chloroquine-resistant strain of *P.falciparum*. A. Quinine B. Mefloquine

Ammonium chloride exerts an antimalarial effect at high concentrations i.e. IC_{50} = 3 mM, which correspond to the concentrations expected to produce significant changes in vacuolar pH based on the Henderson-Hasselbalch equation. Alkalinization of the food vacuole is expected to reduce the accumulation of other lysosomotropic agents due to competition for protons. High concentrations of ammonium chloride (0,1 - 8 mM) had no effect on amantadine sensitivity in the chloroquine-resistant strain, results are displayed as an isobole graph of the interaction of amantadine and ammonium chloride in figure 6.6. This is in contrast to the effect of NH_4Cl on chloroquine sensitivity, high concentrations of ammonium chloride in combination with chloroquine have been reported to be antagonistic (Geary *et al.*, 1989) but parallels the effect seen with mefloquine.

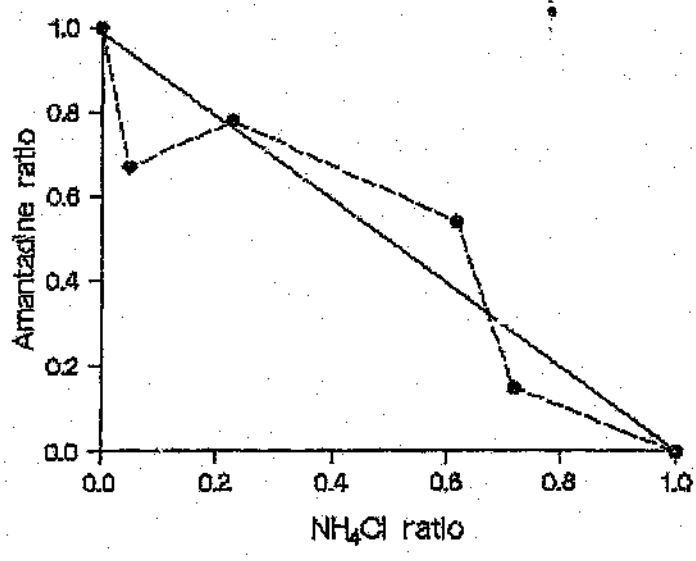


Figure 6.6 : Isobologram of the interaction between high concentrations of ammonium chloride (0.1 - 8 mM) and amantadine in chloroquine-resistant (FCR-3) strain of *P.falciparum*

The lack of correlation of amantadine sensitivity with pH led us to explore whether the amine group was necessary for antimalarial activity. The sensitivity of the parasite to amantadine was found to be dependent on the presence of the amine group on the hydrocarbon cage, since adamantane, the parent compound with identical shape to amantadine but lacking the amine group fails to inhibit parasite growth at concentrations as high as 1 mM see figure 6.7.

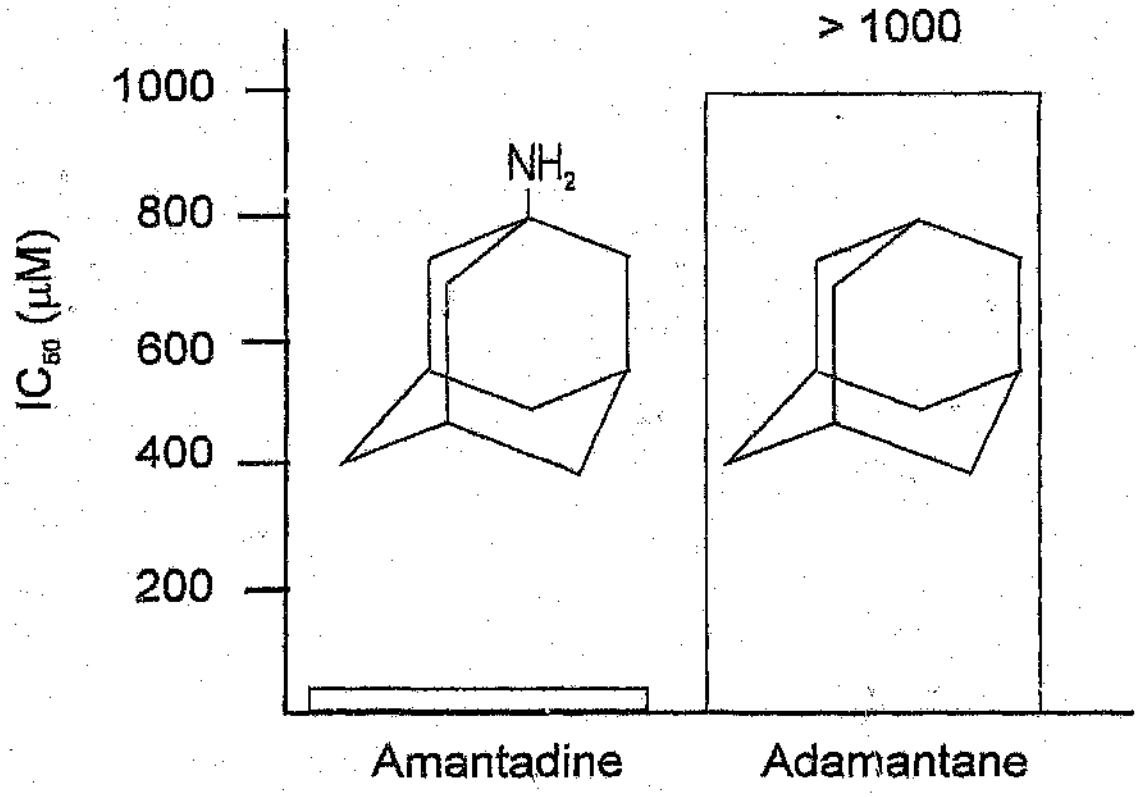


Figure 6.7 : Comparison of IC_{50} of amantadine and adamantane against the chloroquine-resistant (FCR-3) strain of *P.falciparum*

6.3.2 THE EFFECT OF AMANTADINE ON THE PARASITE'S ACIDIC COMPARTMENT

Uninfected red blood cells do not take up acridine orange (results not shown). Parasitized red blood cells rapidly accumulate acidine orange, showing a time dependent quenching of acridine orange fluorescence until steady-state is achieved (fig 6.8). The extent of quenching is proportional to the number of parasites present and the pH of the bathing solution (results not shown). The accumulation of acridine orange is reversed by exposure to agents which dissipate the H^+ gradient.

When the antiamalarial drugs are added they are taken up via the pH gradient and compete for protons forcing accumulated acridine orange out of the parasite and a new steady state is achieved. The magnitude of the change in pH is calculated based on the the amount of acridine orange released. The pattern of acridine orange titration following treatment with chloroquine, amantadine, quinine and mefloquine is shown in figure 6.9.

The quinolines caused a rapid release of acridine orange (i.e. alkalinization of the food vacuole) but with no further reacidification. Amantadine in contrast, caused a release of acridine orange followed by a gradual reuptake at concentrations below 100 μM.

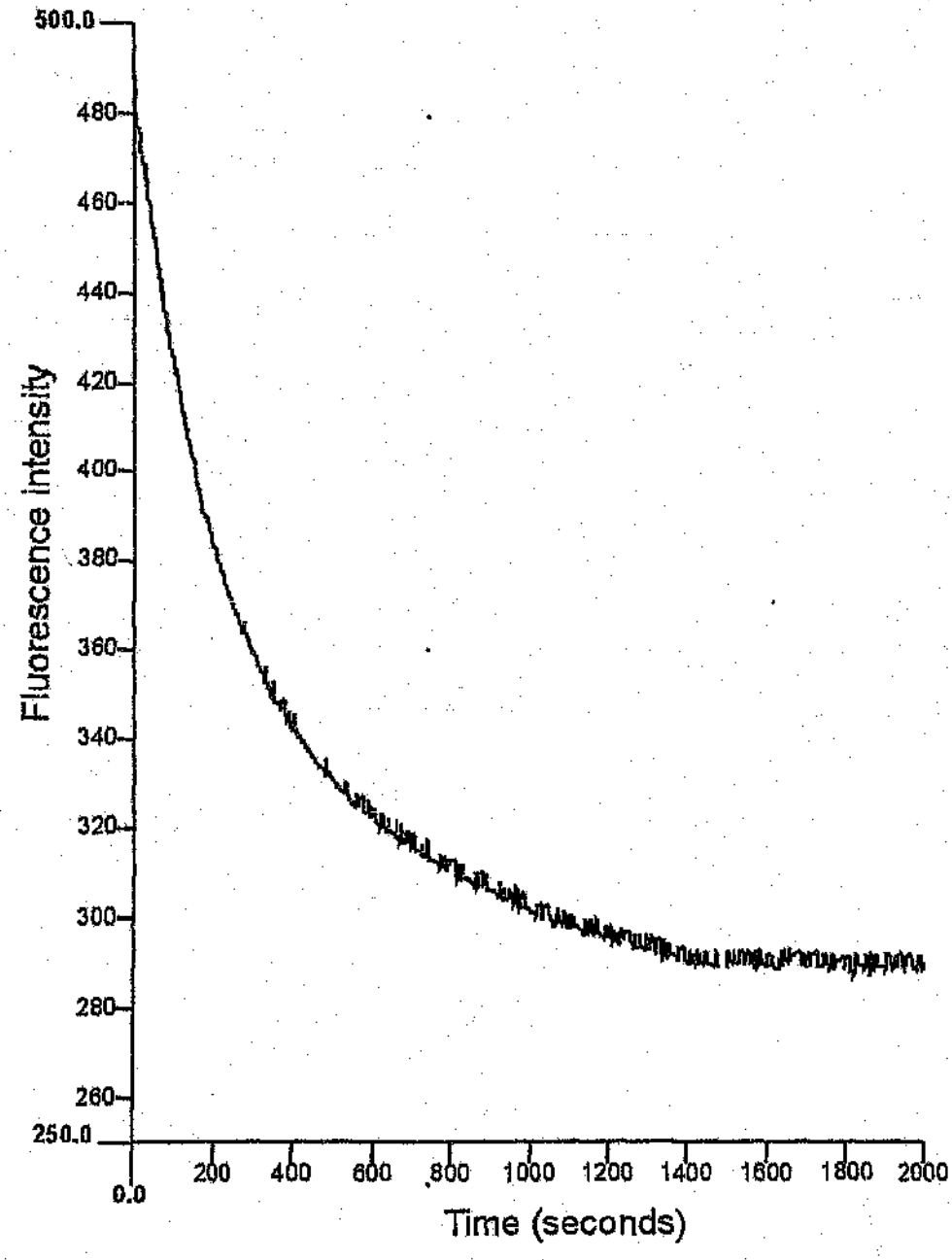


Figure 6.8: Accumulation of acridine orange by parasitized red blood cells

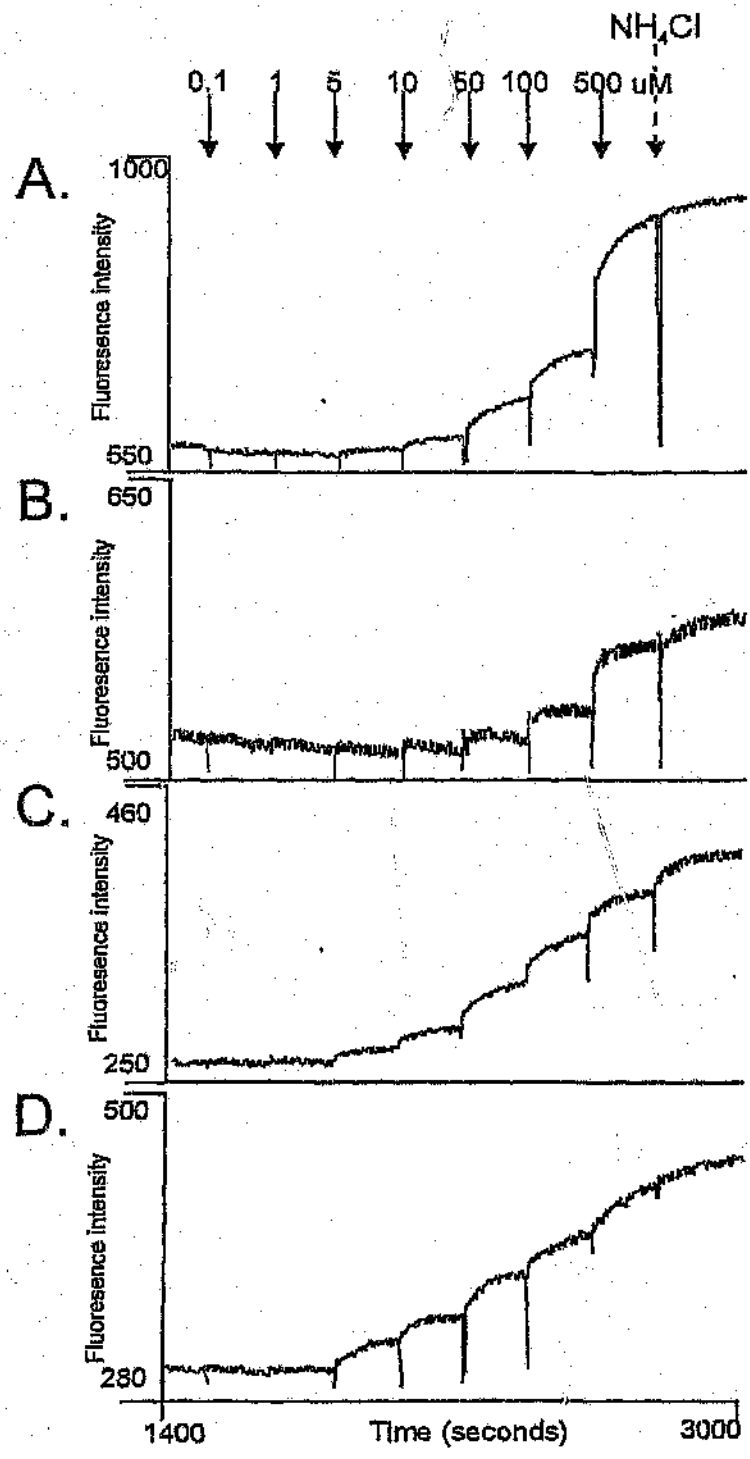


Figure 6.9: Titrating of acridine orange accumulation in *P.falciparum* chloroquine-resistant strain by antimalarial drugs A. Chlorouqine B. Amantadine. C. Quinine. D. Mefloquine

Figure 6.10 and 6.11 compares the dose-response effect chloroquine (dibasic) and monobasic antimalarials and amantadine on the alkalinization of the vacuolar pH in the chloroquine-resistant (FCR-3) stain of *P.falciparum* .

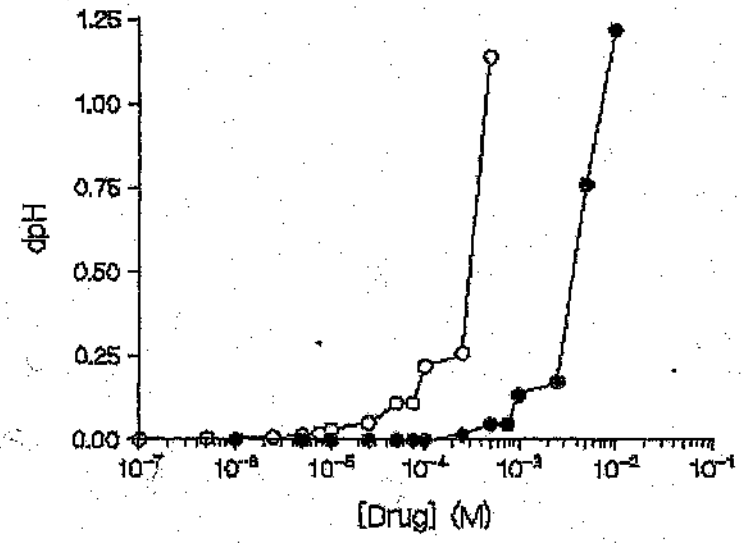


Figure 6.10: Dose-dependent alkalinization of the parasite's food vacuole by chloroquine (O) and amantadine (●) in the chloroquine-resistant (FCR-3) strain

Table 6.1 correlates the antimalarial activity with changes in vacuolar pH in the chloroquine-resistant (FCR-3) strain calculated from the dose response curves. Changes in the vacuolar pH of more than 0.3 units were considered to be significant. The classical antimalarials all resulted in alkalinization in the micromolar range. However, amantadine and ammonium chloride were effective in the millimolar range.

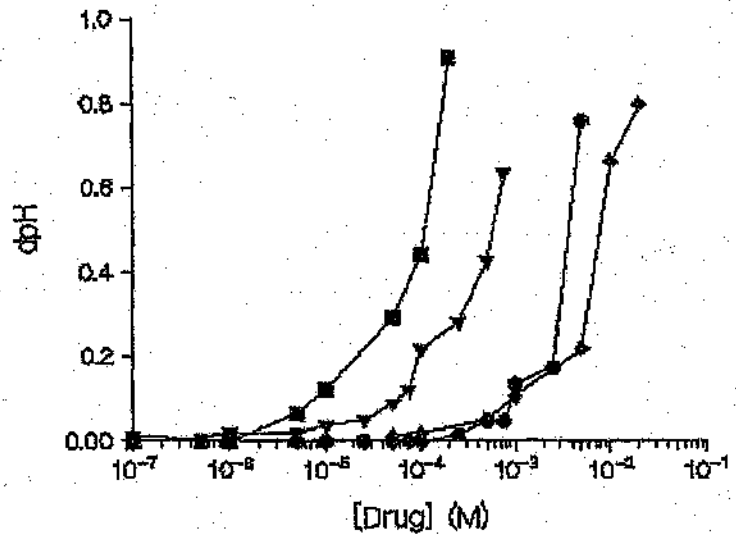


Figure 6.11: Dose-dependent alklainization of the parasite's food vacuole by quinine (▼) mefloquine(■), NH_4Cl (◆) and amantadine (●) in the chloroquine-resistant strain

Table 6.1 : Comparison of the effect of drugs on parasite growth and alkalinization of the food vacuole

COMPOUND	IC_{50}	IC_{ALK}
Amantadine	5.35 ± 1.15 μM	2 ± 0.2 mM
Ammonium chloride	3.1 ± 0.25 mM	3.4 ± 0.3 mM
Chloroquine	176 ± 2.74 nM	2.6 ± 0.4 μM
Mefloquine	15.4 ± 1.21 nM	53 ± 3 μM
Quinine	141 ± 3.6 nM	2.5 ± 0.1 μM

The order of potency in increasing vacuolar pH being MQ>QN>CQ>>AMA≈NH_4Cl.

Although there is no doubt that the antimalarials raised the pH, this effect is observed

at concentrations substantially higher than their respective IC_{50} values, only ammonium chloride's antimalarial activity correlated with its alkalinizing activity.

Figure 6.12 and 6.13 compares the alkalinization effect of chloroquine and amantadine respectively between chloroquine-resistant and chloroquine-sensitive strains. In both cases there was no difference in the alkalinizing effect of the agent in strains showing different chloroquine-sensitivity.

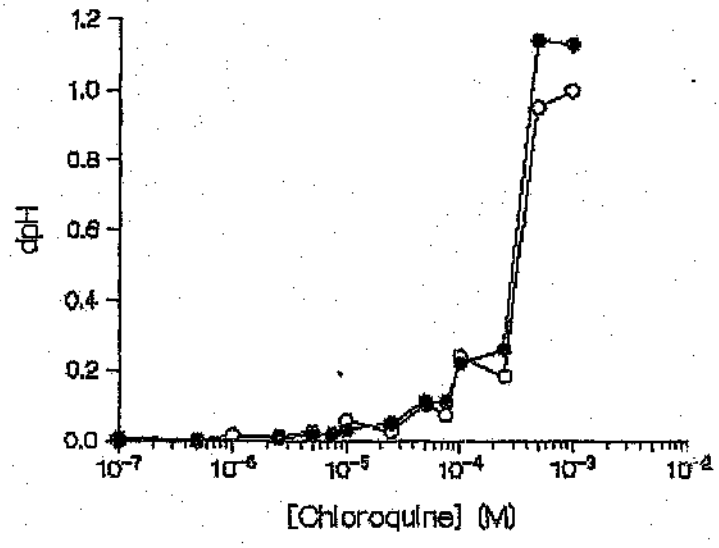


Figure 6.12 : Dose-dependent alkalinization of the parasite's food vacuole by chloroquine in chloroquine-resistant (●) and chloroquine-sensitive (○) strain of *P.falciparum*

Finally, it has been postulated that resistance reversal agents may modulate drug accumulation in resistant strains by altering the pH. To examine this we investigated the effect of verapamil, a classical reversal agent, on alkalinzation of the food vacuole. The IC_{ALK} was ± 100 μM, the concentration capable of resistance reversal is ± 10 μM.

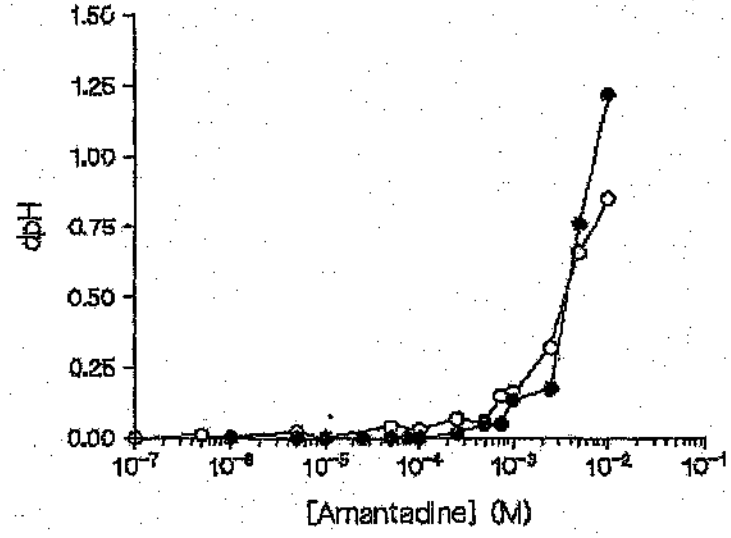


Figure 6.13 : Dose-dependent alkalinization of the parasite's food vacuole by amantadine in chloroquine-resistant (●) and chloroquine-sensitive (○) strain of *P.falciparum*

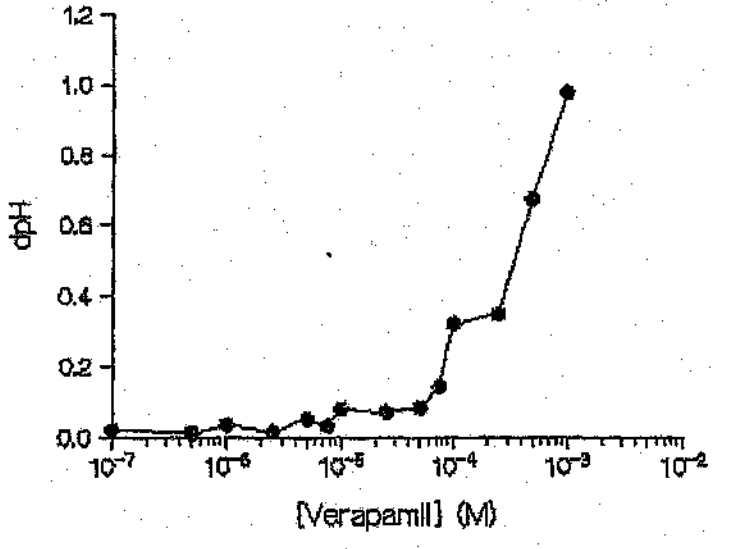


Figure 6.14 : Dose-dependent alkalinization of the parasite's food vacuole by verapamil in chloroquine-resistant (●) strain of *P.falciparum*

6.3.3 THE EFFECT OF pH ON OSMOTIC STABILITY

Amantadine stabilizes the erythrocyte membrane at high concentrations > 1 mM at both pHs (fig 6.15a). The effect of amantadine differs form the effect seen with chloroquine. Chloroquine affords resistance to lysis at pH 7.4 but destabilizes the membrane at acidic pHs (fig 6.15b).

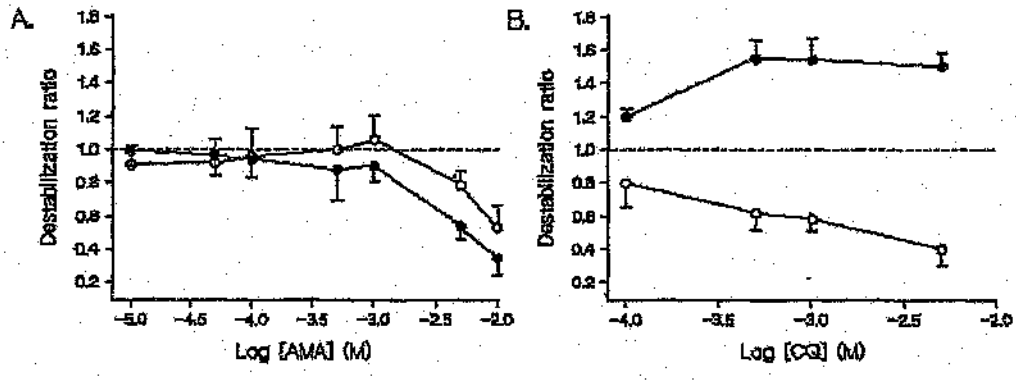


Figure 6.15: Effect of A. amantadine B. chloroquine on osmotic stability of human erythrocytes
● pH 5 ○pH 7.4

6.4 DISCUSSION

The weak base effect predicts that the IC_{50} decreases as the pH increases due to increase in the relative concentration of the unionized species. Both chloroquine and quinine accumulation via the pH gradient correlates with drug sensitivity (fig 6.3 and fig 6.5a). The relationship between drug senstivity and pH differs for amantadine and mefloquine. Mefloquine sensitivity is unrelated to pH (fig 6.5b), possibly reflecting electrogenic accumulation of mefloquine (Vanderkooi *et al.*, 1988). Amantadine accumulates in the food vacuole in both strains, as evidenced by osmotic swelling and alkalinization. However, the antimalarial activity of amantadine appears to involve

factors in addition to the concentration in the food vacuole in the chloroquine-resistant strains (fig 6.4). Nevertheless, the amine group is critical for activity (fig 6.7).

There are two possible interpretations of the unusual relationship between amantadine and pH. The first, that amantadine accumulation occurs by an electrogenic secondary transport process analogous to the mechanism by which catecholamines are actively accumulated by chromaffin granules of the adrenal medulla (Johnson *et al.*, 1982). In this model, initially proposed by Vanderkooi *et al.* (1988) to explain mefloquine accumulation, the drug crosses the membrane in a positive charged, monoprotonated form. This model predicts that drug sensitivity should be independent of external pH, since at physiological pH the charged species of drug is predominant. Mefloquine sensitivity is independent of pH sensitivity as predicted, however, amantadine sensitivity shows pH dependence making electrogenic accumulation of amantadine unlikely. The second possibility is that the target of amantadine is on the outer membrane rather than within the food vacuole. Changes in external pH are expected to influence the relative ionziation of membrane phospholipids and proteins as well as the drug. The relative charge distribution of these components is critical in the interaction of amphiphilic compounds. Amantadine is membrane active, both its antiviral (Pinto *et al.*, 1992) and cerebral effects (Kornhuber *et al.*, 1994) are related to interactions with membrane proteins.

The alkalinizing activity of amantadine equals that predicted by Henderson-Hasselbalch equation and is unrelated to the antimalarial activity of amantadine (table 6.1). However, the alkalinizing effect of the quinolines is greater than that predicted, although still insufficient to account for the antimalarial activity (table 6.1). The increased alkaliniation seen with the quinoline is attributed to increase H^+ leak or to inhibition of the pump or both (Ginsburg *et al.*, 1989).

Drug accumulation in the food vacuole is expected to result in osmotic swelling (De Duve *et al.*, 1974). The increase in the concentration of drug in the food vacuole creates a charge imbalance, which drives anions into the vacuole, the increased osmotic concentration in the lumen of the organelle results in water influx, causing swelling. In the case of amantadine the increased volume results in futher uptake of acridine orange without additional change in the pH_i. The quinolines don't show the transients due to inhibition of the proton pump (Choi and Mego, 1988).

The destructive effect of amantadine could be due to osmotic swelling and the subsequent lysis of the acid compartment releasing hydrolases into the parasite cytosol thereby damaging the cellular machinery. However, amantadine at the concentrations attained in the food vacuole has a stabilizing effect on membranes (fig 6.15), most likely due to its ability to intercalate into the lipid bilayer of the membrane, thereby expanding the membrane allowing the cell to expand to a larger volume before it lyses.

These results suggest that the primary effect of amantadine is unlikely to be disrupting of the integrity of the vacuole membrane.

The pH gradient is implicated in drug resistance. It has been demonstrated that chloroquine-resistant parasites have elevated food vacuole pH relative to the senstitive parasites (Geary *et al.*, 1990). The differences are postulated to be due to impaired functioning of the vacuolar pump mechanism. A Mg^{2+}-dependent ATPase responsible for acidification has been identified in vacuolar membrane of the parasite. It is inhibited by classical proton pump inhibitors as well as by the antimalarial quinine (Choi and Mego, 1988). Inhibition of the pump reduces both the accumulation and susceptibility to chloroquine.

The basis of weakend proton pump activity is open to conjecture. There is no evidence that the proton pump either functions differently or shows an altered sensitivity profile between strains (Slater, 1993). A finding confirmed by our results (fig 6.12 and fig 6.13), which show no difference in the alkalinizing effect of chloroquine or amantadine in different strains despite different levels of chloroquine accumulation. Proton leak and counterion conductance are factors also involved in modulation of vacuolar pH (Bakker-Grunwald, 1992), the possible relationship of these with chloroquine resistance will be discussed in the chapter eight.

6.5 SUMMARY

Amanatadine accumulates in the food vacuole. Factors in addition to pH gradient are involved in the accumulation of the drug in chloroquine-resistant strains. The presence of the amine group on the hydrocarbon cage is essential for the activity of amantadine. Ammonium chloride exerts its antimalarial acitivity by alkalinization of the food vacuole, does not antagonize the effect of amantadine. Amantadine alkalinizes the food vacuole in the millimolar range, however, since antimalarial activity is the micromolar range, alkalinization of the food vacuole is not the primary action of the drug.

CHAPTER SEVEN - MEMBRANE

7.1 INTRODUCTION

Malarial parasites interact with the red cell membrane at a number of crucial stages during development; firstly in the invasion of parasites into red blood cells (Dluzewski *et al.*, 1985); secondly, in modifications of the erythrocyte membrane which occurs in order to facilitate parasite growth, such as the transference of nutrients and waste products (Cabantchik, 1989); thirdly, in the binding or cytoadherence of parasitized erythrocytes to endothelial cells in a process called 'sequestration' (Berendt *et al.*, 1994), and finally, in the ability of parasitized cells to bind uninfected red blood cells in a process known as 'rosetting' (Hasler *et al.*, 1990). Parasitized red blood cells also become targets for protective immunity, and in this respect the membrane provides the interface between the parasitized cell and the immunological system of the host and can bind macrophages, neutrophils and lymphocytes (Kwiatkowski, 1992).

The membrane of the parasitized red blood cell differs in many respects from the parent membrane. The phospholipid and fatty acid composition of the parasitized red blood cell is substantially different from uninfected erythrocyte (Maguire and Rosenthal, 1990; Vial *et al.*, 1990). The asymmetric arrangement of the phospholipids is altered (Gupta and Mishra, 1981). The membrane of infected cells becomes more permeable to a wide range of substrates, a number of novel transport channels have

been identified, the biochemical nature of them has yet to be characterised (Ginsburg, 1994).

Amantadine, can interact strongly with the membrane resulting in changes in the structural properties of the membrane (Colman *et al.*, 1977; Herrmann *et al.*, 1985).

In this section we investigated changes in membrane fluidity and permeability following exposure of chloroquine-resistant and chloroquine-sensitive parasites to amantadine and finally the effect of membrane associated amantadine on development of the parasite.

7.2 EXPERIMENTAL PROCEDURE

7.2.1 THE EFFECT OF AMANTADINE ON MEMBRANE FLUIDITY (Deguercy *et al.*, 1986)

Fluorescent probes partition to varying degrees into the hydrophobic phase of the lipid bilayer and can be used to detect, by fluorescence, subtle changes in the molecular dynamics and physical state of lipid molecules in the vicinity of the probe (Slavik, 1982).

7.2.1.a Sample preparation

Parasites at the mature trophozoite stage were concentrated using the gelatin floatation technique. Infected or uninfected erythrocytes were washed three times in

cold isotonic phosphate buffered saline, pH 7.4, and were suspended at a concentration of 6 x 10^6 cells/ml. The parasitemia of the infected cells was between 50 - 70 %.

7.2.1.b Cell labelling with ANS

Anilinonaphthalene sulfonate (ANS) was dissolved in PBS buffer to give a final concentration of 30 μM per sample. ANS was added to aliquots of cells. Fluorescence polarization values were assessed 5 min after the addition of ANS.

7.2.1.c Fluorescence polarization measurements

Dye uptake was measured via fluorescence intensity (excitation 385 nm, emmission 483 nm) as a function of time. Fluorescence anisotropy and polarization values were determined at room temperature in a stirred cuvette, using spectophotometer in the "T" configuration and software provided by Perkin Elmer. The excitation wavelength used was 385 nm and the wavelength of emission was 483 nm. The excitation and emmission slits were 5 nm and 10 nm respectively. Proper corrections were applied for the fluorescence of the buffer with only ANS and buffer with cells only.

7.2.2 THE EFFECT OF AMANTADINE ON MEMBRANE PERMEABILITY (Kutner *et al.*, 1982)

During the intra-erythrocytic development of the parasite, the red blood cell show increased permeability to a variety of low molecular weight solutes (Cabantchik, 1989). The properties of the pathway suggest it may be a pore or channel (Kirk *et al.*,

1994). A simple, semi-quantitative hemolysis method was used to monitor the effect of amantadine on the transport of sorbitol, a sugar, into infected cells.

7.2.2.a Sample preparation

Cultures of parasitized red blood cells which had reached the trophozoite stage were enriched using the gelatin floatation technique. Parasites were washed 3 times in PBS supplemented with 10 mM glucose. Parasites were used immediately.

7.2.2.b Iso-osmotic lysis assay

Iso-osmotic solutions of sorbitol were prepared by dissolving the compounds to a concentration of 300 mM in water containing 10 mM HEPES (pH 7.4). The time course of hemolysis was commenced by the addition of malaria infected cells to the sorbitol solution (pre-warmed to 37°C) to give a final haematocrit concentration of 0.5%. Aliquots of suspension were sampled at regular intervals into microcentrifuge tubes and spun for 20 s. The supernatant solution was removed, and the haemoglobin in this solution, and hence the degree of haemolysis, estimated from the absorbance at 540 nm. Normal, uninfected erythrocytes, incubated under the same conditions remained stable (i.e. did not lyze) for at least 1 hour. Hemolysis may be attributed to the net influx of the compound into the infected cells via the malaria induced transport system.

7.2.3 THE EFFECT OF MEMBRANE ASSOCIATED AMANTADINE ON PARASITE DEVELOPMENT

7.2.3.a Erythrocyte preparation

Erythrocytes were washed and treated with different concentrations of amantadine for 8 hours at 37°C. Prior to parasite treatment erythrocytes were either washed to remove membrane associated amantadine or allowed to settle and amantadine containing medium was aspirated off (control).

7.2.3.b Isolation of merozoite

Erythrocytes harbouring parasites at mature stages (i.e. trophozoites and schizonts) were concentrated from culture using the gelatin floatation. After separation the cells were washed twice in medium and cultured until late schizonts. Prior to invasion assay, cells were pelleted by centrifugation.

7.2.3.c Merozoite invasion assay

Pre-treated erythrocytes were suspended in hypoxanthine free medium and samples (200 µl) were aliquoted into 96 well microtitre plate. Parasitized erythrocytes (schizont-rich) were added to wells. Parasite growth was assessed using the hypoxanthine method as previously described.

7.3 RESULTS

7.3.1 THE EFFECT OF AMANTADINE ON MEMBRANE FLUIDITY

The kinetics of ANS accumulation in parasites was determined. Uptake of ANS was very rapid. When steady-state was reached, there was judged to be no significant difference between chloroquine-resistant and chloroquine-sensitive parasites (figure 7.1). Data shown in figure represents results of typical experiment.

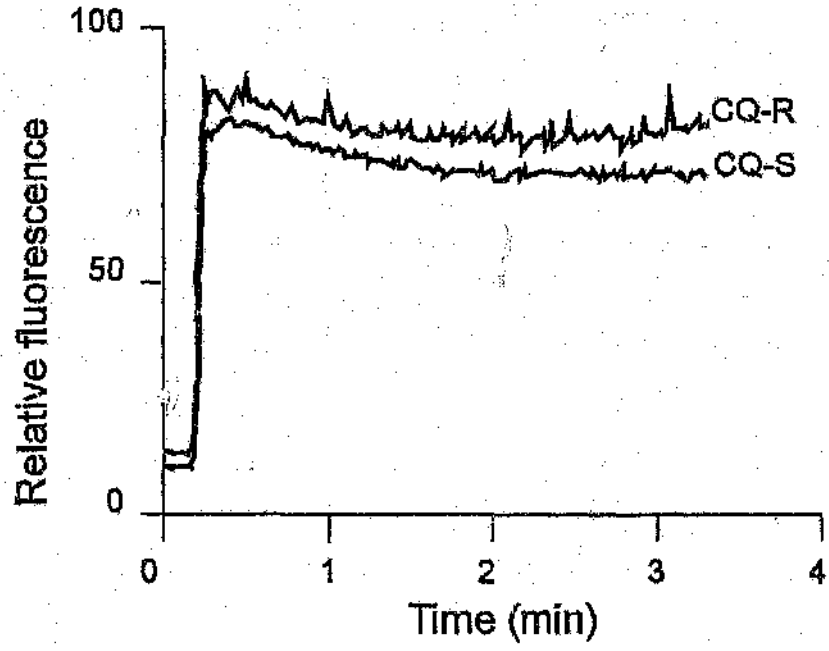


Figure 7.1: Accumulation of ANS by CQ-R (FCR-3) and CQ-S (3D7a) parasites as a function of time

The anisotropy decreased significantly with the time of incubation (results not shown). This reflects movement of the probe from the plasma membrane to intracellular sites over time.

The polarization and anisotropy value of the ANS is higher in uninfected erythrocytes compared to parasitized erythrocytes (see figure 7.2). Confirming that there is an increase in fluidity as a result of infection, as previously reported (Deguercy *et al.*, 1986 ; Koppaka *et al.*, 1989).

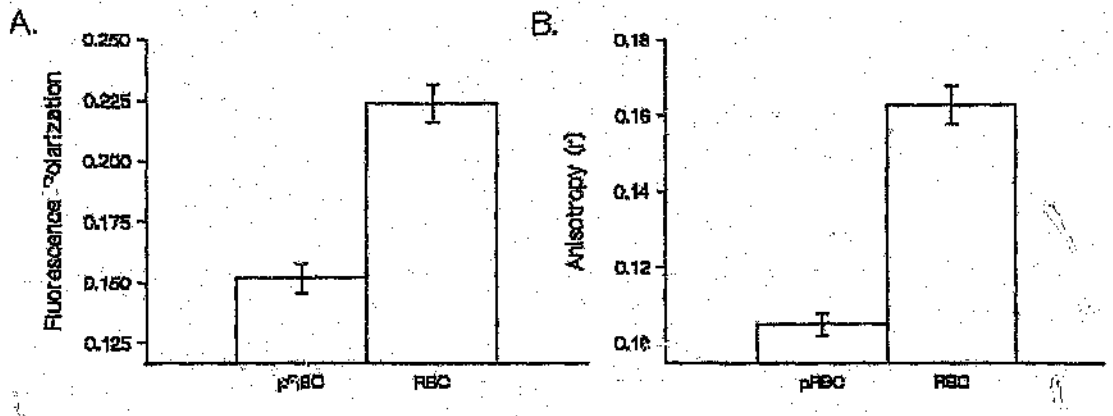


Figure 7.2 : Fluorescence polariztion values in parasitized red blood cells and uninfected cells and anisotropy (r) values

Benzyl alcohol, a known membrane fluidizer (Sinicrope *et al.*, 1992), significantly altered membrane fluidity of parasitized erythrocytes membrane as assessed by r values of ANS. In contrast a 30 min exposure to 100 μM amantadine did not affect fluorescence anisotropy of parasites (figure 7.3). The addition of 100 μM verapamil did not have an effect of membrane fluidity as assessed by ANS - results not shown.

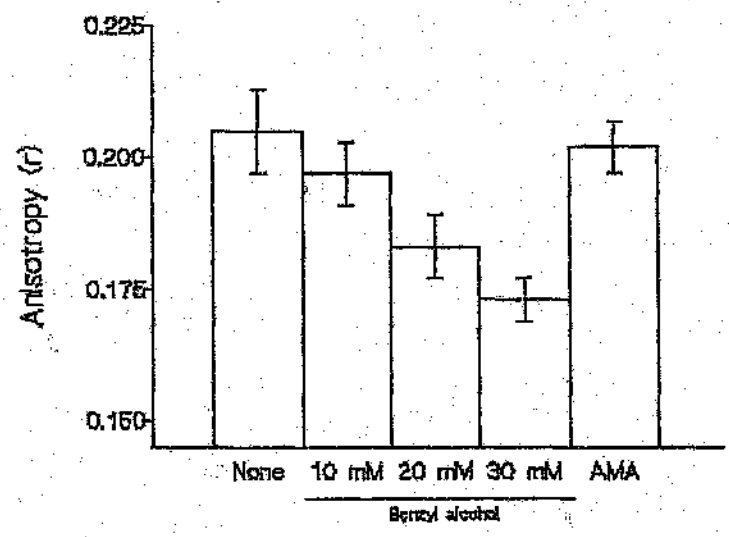


Figure7.3: Anisotropy (r) values following treatment with 100 μ M amantadine and benzyl alcohol (a membrane fluidizer)

7.3.3 THE EFFECT OF AMANTADINE ON MEMBRANE PERMEABILITY

When parasitized red blood cells were suspended in an iso-osmotic solution of sorbitol, they lyzed. Lysis was due to entry of the solute through the parasite induced permeation pathways and the ensuing osmotic swelling exceeding the lytic volume of the red blood cell - see figure 7.4

The uptake of sorbitol and subsequent lysis can be ascribed to an active transport mechanism in the parasite. Lysis is not due to passive diffusion since hemolysis is absent at 4°C - see figure 7.5.

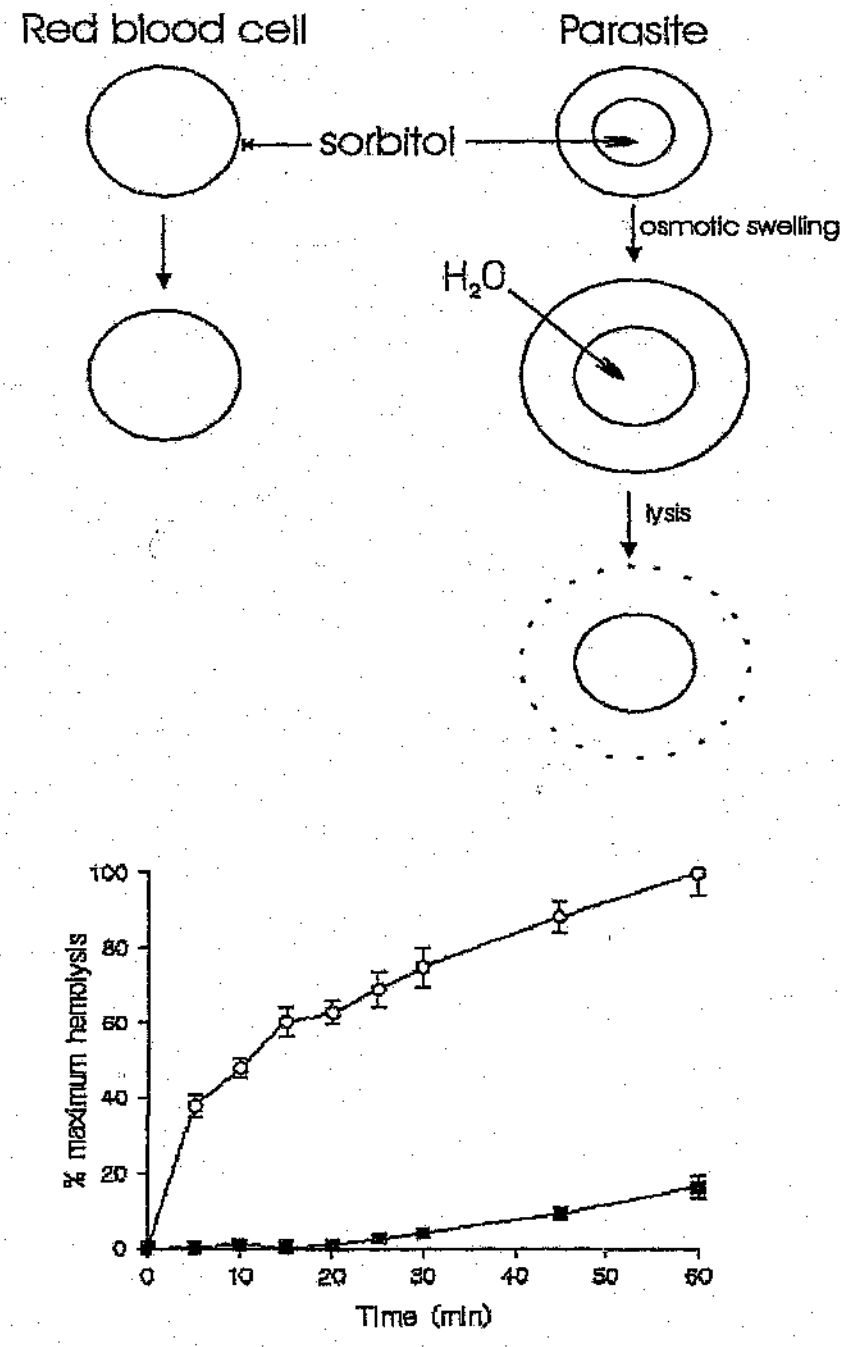


Figure 7.4 : Time dependence of sorbitol lysis by *P.falciparum* infected (O) and uninfected (■) red blood cells

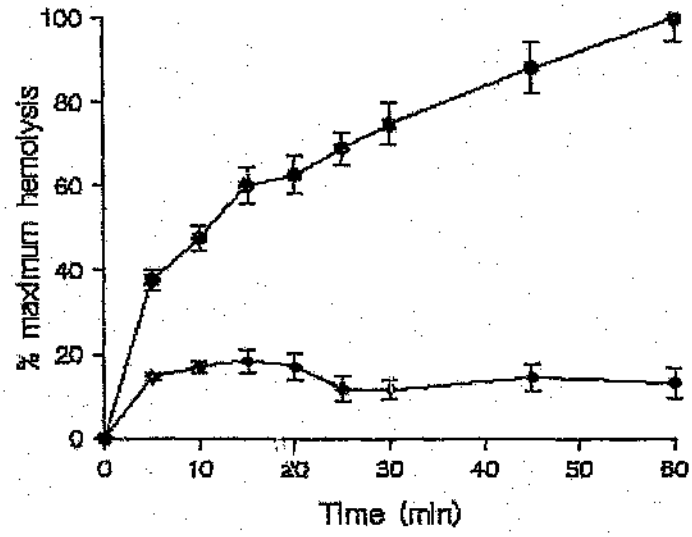


Figure 7.5 : Temperature dependence of sorbitol lysis ● at 37°C and ◆ at 4°C

The effect of amantadine on the transport of the sugar alcohol, D-sorbitol, into the chloroquine-resistant (FCR-3) strain of *P.falciparum* is shown in figure 7.6. Amantadine when present in the solute showed a slight protective effect against hemolysis. This inhibition of lysis probably reflects the membrane stabilising activity of amantadine (demonstrated in chapter six) rather than a block of the permeation pathways. Amantadine had no effect on the rate of hemolysis following pre-treatment with amantadine for 30 minutes - this result is consistent with the idea that amantadine does not block sorbitol influx. However, prolonged treatment with amantadine 2 and 4 hours produced dramatic decreases in the rate of hemolysis - see figure 7.7. These effects may be secondary effects due to parasite death, however, the data obtained from time dependence studies (chapter five) suggest that the indirect inhibition of parasite permeation pathways occurs prior to parasite death.

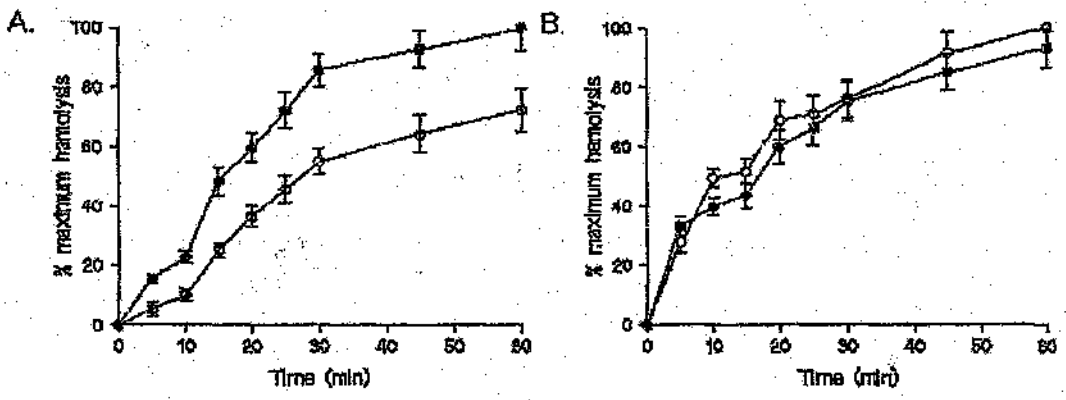


Figure 7.6: The time course for haemolysis of the chloroquine-resistant strain (FCR-3) suspended in iso-osmotic solutions of sorbitol in the absence (●) and presence (○) of 100 μM of amantadine. A. Present during exposure B. Pre-treated with drug for 30 min

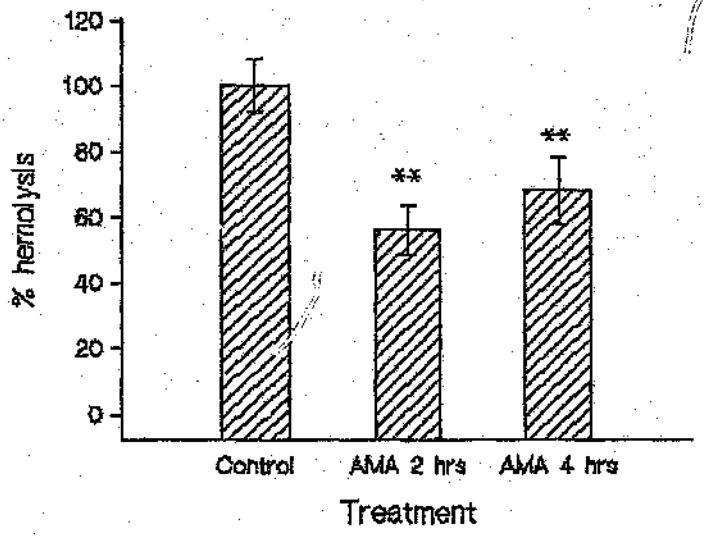


Figure 7.7: The time course of haemolysis of untreated (control) and amantadine pre-treated parasites suspended in iso-osmotic solutions of sorbitol ($p < 0.001$)

7.3.3 THE EFFECT OF MEMBRANE ASSOCIATED AMANTADINE ON PARASITE DEVELOPMENT

Richman *et al.* (1981) identified two intracellular components of amantadine. The first is a cell associated (lysosome and cytosol) component which resists elution by washing and the second component is membrane associated and is sensitive to elution. The antimalarial activity of amantadine was lost upon the removal of the membrane-associated component (figure 7.8).

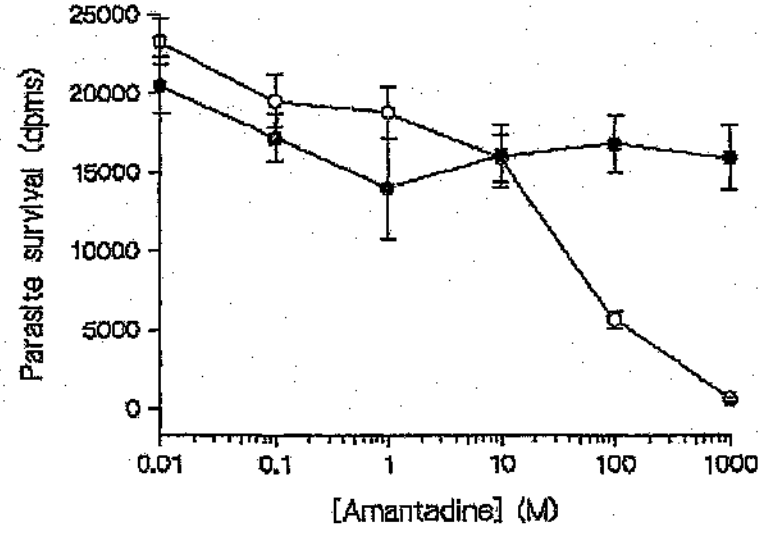


Figure 7.8: Antimalarial activity following removal of amantadine (●) and control (○)

7.4 DISCUSSION

Amantadine has been reported to both perturb the organisation and motional properties of phospholipids in the bilayer (Herrmann *et al.*, 1985; Colman *et al.*,

1977) as well as stabilise clathrin-coated membrane vesicles (Phonphok and Rosenthal, 1991) - the effects of amantadine on bilayer structure and stability are likely to be complex and dependent on both intrinsic and environmental factors. The results of are study (figure 7.3) indicate that amantadine does not markedly perturb the bilayer system in the host cell membrane of the parasitized erythrocyte as a whole. Nevertheless, the component of amantadine associated with the membrane appears to be critical for the antimalarial activity of amantadine. The orientation of the bilayer partitioned amantadine is such that the hydrophobic cyclic region is located between the phosphate and ester linkages of the phospholipids with the NH_3^+ group protruding into the water space (Duff *et al.*, 1993) - see figure 7.9.

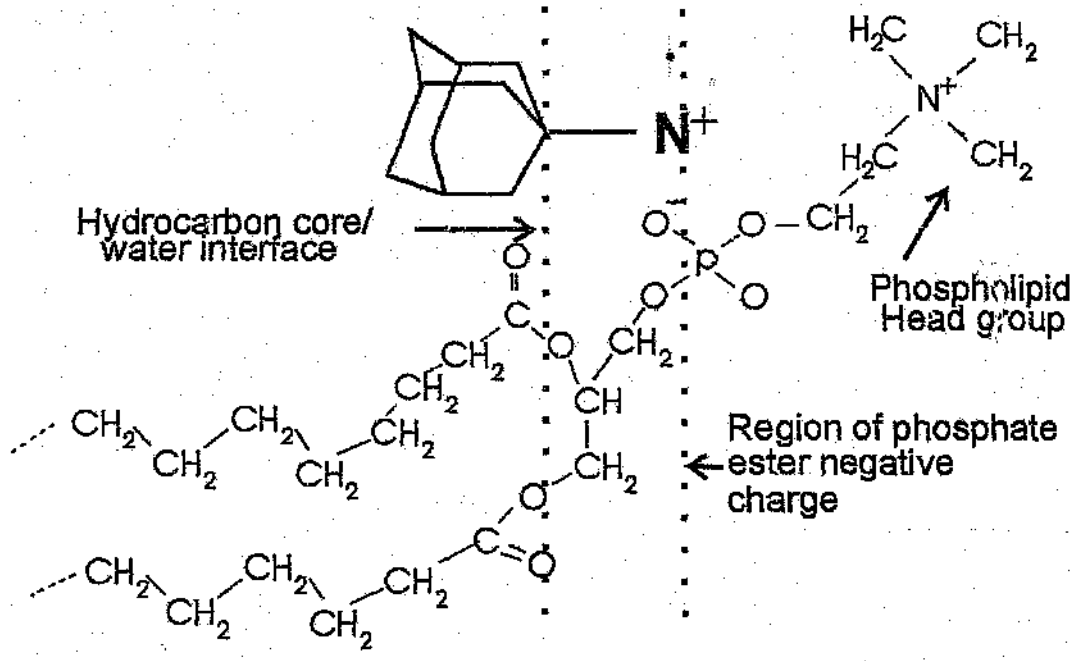


Figure 7.8: Orientation of amantadine in the phospholipid membrane

The location of amantadine near the hydrocarbon core/water interface would allow both hydrophobic interactions with phospholipid fatty acyl chains and electostatic bonding between the charged side group and the anionic oxygen of the phosphate moiety. By altering the physical properties of the membrane - amantadine may perturb the conformation of integral proteins and/or energetics associated with their functional activity (e.g. ion conductance, ligand binding) - see figure 7.10 (Mason, 1993).

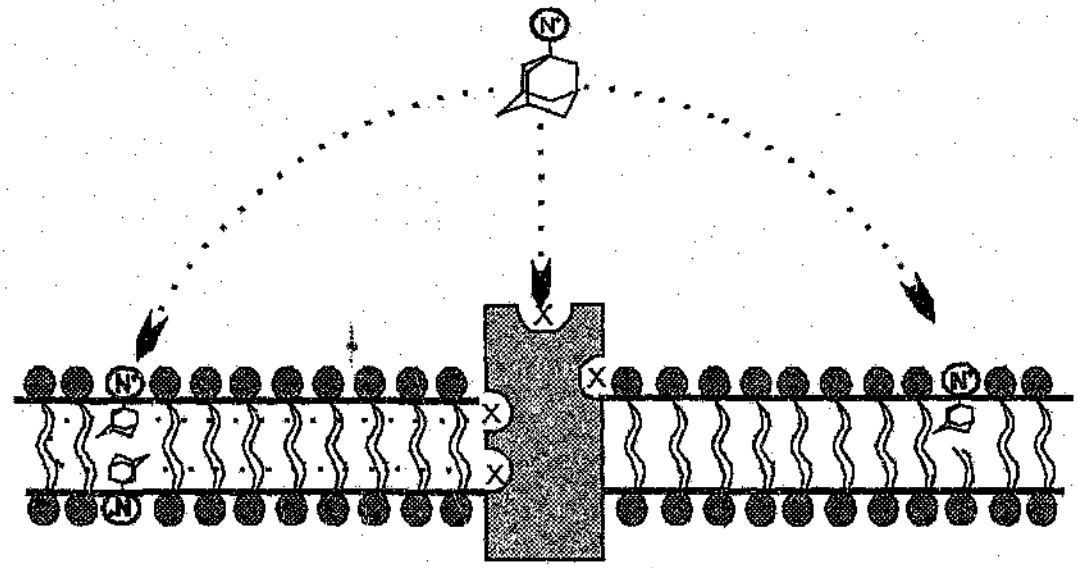


Figure 7.9: Drug interaction with membrane

Variability in membrane composition is expected to dramatically affect the membrane partitioning of the drug and thus its effects. This is of particular significance since it has been reported that resistance to chloroquine and quinine is correlated with the relative content of acidic phospholipids in the infected red blood cell membranes, while mefloquine (and therefore amantadine) is inversely related. Chloroquine resistance has

also been found to be inversely related to the content of cholesterol, while sensitivity to mefloquine (and therefore amantadine) decreases with increased cholesterol concentration (Shalmiev and Ginsburg, 1993).

The parasite carries out major structural renovations to the basic architecture of the host membrane proteins (Elmendorf *et al.*, 1992). Plasmodial proteins are thought to mediate nutrient uptake, ionic balances and cytoadherence in the parasite. The details of the majority of these parasite-derived proteins are unknown. In this study we investigated the effect of amantadine on the parasite induced permeation pathways - the increased permeability is mediated by pathways with characteristics unlike those of the transporters of the normal erythrocyte but showing functional similarities to chloride channels in other cell types (Kirk *et al.*, 1992). Based on the ability of amantadine to block numerous ion channels it was postulated that amantadine may block nutrient uptake and therefore inhibit parasite development. However, the results indicate amantadine does not block this transport mechanism *per se* - since incubation with amantadine for 30 minutes does not result in inhibition of sorbitol influx. However, amantadine appears to have an indirect effect on sorbitol influx - since there is a marked reduction (50 %) of sorbitol influx 2 hours after treatment with amantadine. This inhibition is unlikely to simply reflect secondary toxicity since treatment of parasites with amantadine for 2 hours is insufficient to kill the parasite.

The kinetics of the block suggest that the effect of amantadine on permeation pathways may be related to parasite protein secretion. The mechanism by which the trophozoite brings about the export of parasite-derived proteins and newly synthesised phospholipids through its plasmalemma and across the parasite vacuole membrane to locations within the cytoplasm of the infected erythrocyte and beyond is still unclear. Multiple trafficking mechanisms have been identified (Gormley *et al*, 1992).

7.5 SUMMARY

The membrane associated component of amantadine appears to be critical for antiamalarial activity of amantadine. Amantadine does not significantly alter the fluidity of the red blood cell membrane overall. Amantadine does not inhibit the permeation pathways responsible for nutrient uptake in the parasite directly, however, amantadine blocks the pathway indirectly.

CHAPTER EIGHT - RESISTANCE REVERSAL

8.1 INTRODUCTION

Chloroquine-resistance in malaria is associated with a 40-50 fold higher efflux of the drug in comparison to sensitive isolates (Krogstad *et al.*, 1987). Resistance can be reversed *in vitro*, by the addition of pharmacological agents that inhibit P-glycoprotein (PgP) (Martin *et al.*, 1987). P-glycoprotein is a 170 kDa membrane protein overexpressed in MDR (multidrug resistant) mammalian cancer cells. Genes encoding members of the PgP family have been isolated from the malaria parasite and variations in PgP copy number and/or levels of expression have been implicated in drug resistance.

In this section the chemosensitizer character of amantadine is investigated by studying the effect of amantadine on chloroquine accumulation in a chloroquine-resistant and chloroquine-sensitive strain of *P.falciparum* using radiolabelled drug. As well as, the effect of amantadine on P-glycoprotein function, using rhodamine 123 (a fluorescent dye) known to be a substrate for PgP (Kessel *et al.*, 1991). Throughout this section we employed verapamil as a reference compound. It has been shown that verapamil inhibits the PgP associated, energy dependent drug efflux common to MDR cells. It is a potent and effective antagonist of resistance, to a number of drugs, in MDR cell lines *in vitro* and chloroquine-resistant *P.falciparum in vitro*.

8.2 EXPERIMENTAL PROCEDURE

8.2.1 THE EFFECT OF AMANTADINE ON CHLOROQUINE UPTAKE

8.2.1.a Parasite preparation

Parasites at the mature trophozoite stage were concentrated using the gelatin floatation technique. Infected or uninfected erythrocytes were washed three times in cold isotonic phosphate buffered saline, pH 7.4, and were suspended at a final hematocrit of 5% in PBS supplemented with glucose. The parasitemia of the infected cells was between 50 - 70 %.

8.2.1.b Measurement of drug accumulation

Incubation was carried out for the specified length of time in medium containing 100 nM ^{3}[H]-chloroquine (1 µCi/ml) (donated by Prof Warhurst), together with other drugs as required. All reactions were carried out at 37 °C. Exposure was terminated, at the desired time point, by centrifugation (12 000 rpm for 10-20 sec) of a 0.5 ml aliquot of cells in a 1.5 ml eppindorf containing 0.3 ml dibutyl phthalate (DBPH). The effect of drugs was measured by pre-incubating the cells for 15 min, at 37°C, in the presence of the selected agents, before the addition of chloroquine. An additional 30 min was allowed for equilibration of the antimalarial before centrifugation and determination of label in the pellet containing the parasite by scintillation counting.

8.2.1.c Sample processing

After centrifugation the cells containing the labelled chloroquine were sedimented from the medium containing the remaining labelled chloroquine, which remained above

the oil layer. The eppindorf was frozen by immersing in liquid nitrogen. The cell pellet was removed from the centrifuge tube by cutting off the tip of the centrifuge tube (containing the cell pellet) with dog toe clippers. The tube containing the cell pellet was placed in scintillation vial. The cells were lysed by vortex mixing the pellet with 200 µL of distilled water. The lysate was digested with Triton-X overnight, decolourized with H_2O_2 and acidified with glacial acetic acid (Vanderkooi *et al.*, 1988).

8.2.1.ii Data analysis

The concentration of labelled chloroquine in the pellet containing the parasites was calculated from the radioactivity by use of a calibration curve prepared from solutions of known concentration. The number of parasites in the pellet was estimated from the parasitemia and haematocrit. Chloroquine concentration is expressed as fmol/million parasitized red blood cells.

8.2.2 THE EFFECT OF AMANTADINE ON P-GLYCOPROTEIN FUNCTION

The functional release of rhodamine 123 (R123) is correlated with the resistance level to chemotherapy in human cancer cell lines. The use of fluorescent dyes that behave as chemotherapeutic drugs without any deleterious effects on cell biology and viability is an alternative to a functional study with the drug itself.

8.2.2.a Sample preparation

Cultures of parasitized red blood cells which had reached the trophozoite stage were enriched using the gelatin floatation technique. Parasites were washed 3 times in pre-warmed PBS supplemented with 10 mM glucose. Parasites were used immediately.

8.2.2.b Rhodamine 123 labelling

A stock solution of R123 (1 mg/ml) was made in distilled water and stored at 4°C. Cells were suspended in medium with R123, to give a final dye concentration of 10 µg/ml and final hematocrit of 1.5 % for 60 min at 37°C. The cells were then washed with 3 ml cold PBS and centrifuged at 1500 rpm for 5 min. The cells were resuspended and maintained at 37°C for 30 min in drug free medium.

8.2.2.c Harvesting parasites

Exposure was terminated at specified time points and the parasite pellet collected by centrifugation. Cell-associated R123 was extracted with 3 ml butanol. The fluorescence intensity was determined using the fluorescence spectrophotometer with settings at 515 nm excitation (slit width 5 nm) and 535 nm emmission (slit width 5 nm) using 530 nm emmission filter. The fluorescence signal was proportional to the concentration of R123.

8.2.2.d Data analysis

The R123 release index (RRI) was determined. RRI is defined as the mean R123 fluorescence intensity at maximum dye incorporation (60 min after incubation of cells) in medium with R123) divided by the mean R123 fluorescence intensity 30 min after the incubation of the cells in R123 free medium. The mean fluorescence intensity

obtained for autofluorescence was subtracted from each mean fluorescence intensity in the calculation of the R123 index. The variation was less than 10 % of the mean.

8.3 RESULTS

8.3.1 DOSE RESPONSE OF CHEMOSENSITIZERS *IN VITRO*

Resistance reversal agents have intrinsic antimalarial activity in the micromolar range and exhibit the inverse resistance phenomenon as observed with amantadine, i.e. chloroquine-resistant strains are more sensitive than chloroquine-sensitive strains - see fig 8.1. However, like mefloquine, the magnitude of this effect is significantly smaller than for amantadine : the resistance ratio for verapamil is 3.5 compared to the 59.4 for amantadine.

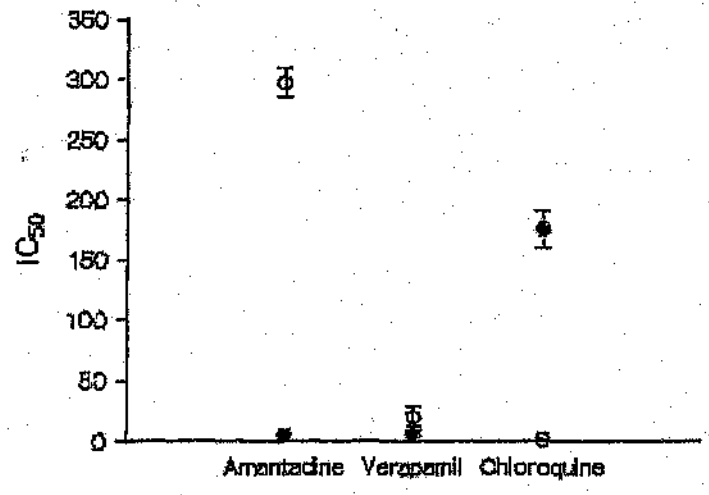


Figure 8.1: Comparison of the antimalarial activity of amantadine and chemosensitizers against chloroquine-resistant (●) and chloroquine-sensitive (O) strains of *P.falciparum*

The mechanism of the intrinsic antimalarial activity of reversal agents is unknown. When amantadine is combined with verapamil the drug interaction is additive (fig 8.2) - suggesting the mechanisms are distinct, since an identical target would be expected to result in an antagonistic interaction.

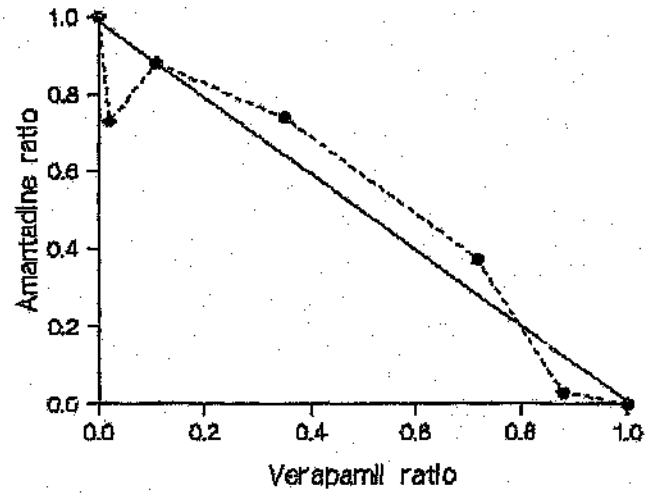


Figure 8.2 : Isobologram depicting the interaction of amantadine with the calcium channel blocker verapamil against the chloroquine-resistant (FCR-3) strain

8.3.2 THE EFFECT OF AMANTADINE ON CHLOROQUINE UPTAKE

The uptake of chloroquine is plotted as a function of time in figure 8.3 for uninfected red blood cells and *P.falciparum* infected red blood cells. The time required for half maximal uptake of chloroquine by infected red blood cells was about 1 min, and maximum levels were attained within 5 min as previously described (Geary *et al.*, 1986). The uptake of chloroquine by uninfected red blood cells was minimal, but continued to increase over the time course of the experiment.

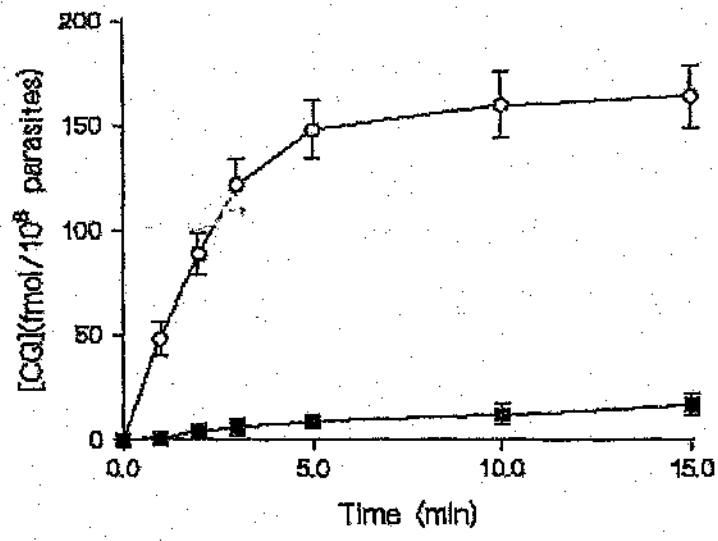


Figure 8.3: Time course of chloroquine uptake by *P.falciparum* (FCR-3) infected (○) and uninfected (■) red blood cells

The chloroquine-resistant parasites accumulated less chloroquine than the chloroquine-sensitive parasites (fig 8.4) as previously reported (Fitch, 1970, Geary *et al.*, 1986, Ginsburg and Stein, 1991, Bray *et al.*, 1992). This difference is believed to be the basis for chloroquine-resistance.

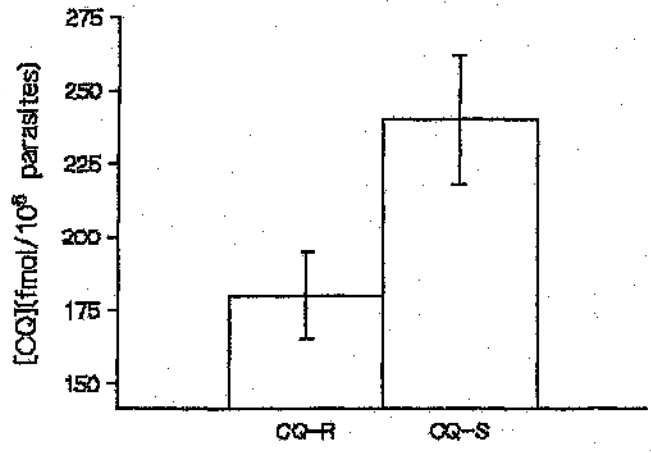


Figure 8.4 : Steady state levels of chloroquine after incubation at 37°C for 60 min in chloroquine-resistant (FCR-3) and chloroquine-sensitive (3D7a) strains of *P.falciparum* ($P < 0.001$)

The isobologram of amantadine and verapamil in combination with chloroquine are shown in fig 8.5a and fig 8.6a. Verapamil is synergistic only against the chloroquine-resistant strain while amantadine is synergistic with chloroquine irrespective of chloroquine sensitivity. Accumulation of radiolabelled chloroquine, followed the pattern seen in the isobolograms. Verapamil enhanced chloroquine uptake in the chloroquine-resistant parasites but had no effect on chloroquine uptake in the chloroquine-sensitive parasite (fig 8.5b). Amantadine increased chloroquine accumulation to the same extent in both strains (Figure 8.6b). The accumulation in the sensitive strain was shifted, corresponding to the shift in the dose response curve. The increase in chloroquine accumulation at reversal concentrations following amantadine treatment was smaller (160 %) than the increase for verapamil (250 %) at 10 μM. The increase in chloroquine accumulation following amantadine treatment may be insufficient to account for the change in drug sensitivity.

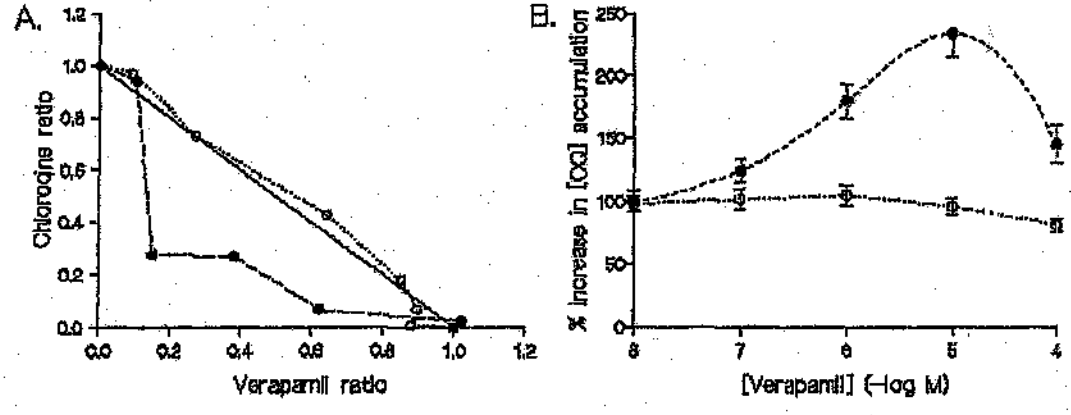


Figure 8.5 : Effect of verapamil-chloroquine combination against chloroquine-resistant (●) and chloroquine-sensitive (○) strains of *P.falciparum in vitro*. A. Isobologram showing drug interaction between chloroquine and verapamil. B. Accumulation of radiolabelled chloroquine in the presence of different concentrations of verapamil

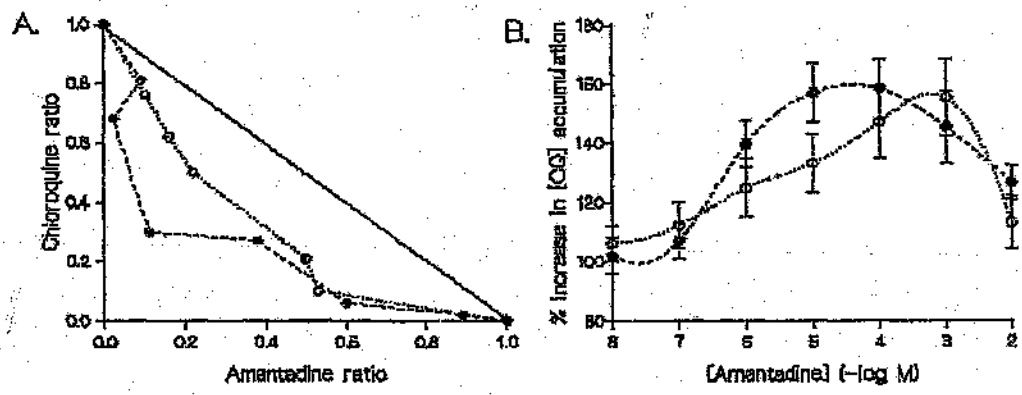


Figure 8.6 : Effect of amantadine-chloroquine combination against chloroquine-resistant (●) and chloroquine-sensitive (○) strains of *P.falciparum in vitro*. A. Isobologram showing drug interaction between amantadine and verapamil. B. Accumulation of radiolabelled chloroquine in the presence of different concentrations of amantadine

8.3.3 THE EFFECT OF AMANTADINE ON P-GLYCOPROTEIN FUNCTION

Both uninfected and infected erythrocytes incorporated the fluorescent dye with a concomitant increase in fluorescence which reached a plateau at 60 min of incubation (fig 8.7). For uninfected erythrocytes, the staining remained stable after withdrawal of the dye. A rapid decrease in R123 fluorescence was observed in parasitized red blood cells following dye withdrawal (fig 8.7). The release of R123 by the parasite exhibited different kinetics from the release associated with MDR phenotype. P-glycoprotein dependent pumping activity is associated with a rapid and complete loss of fluorescence, within 15 minutes of incubation in a dye-free medium. P-glycoprotein independent mechanisms are characterised by a slow and incomplete decrease in fluorescence.

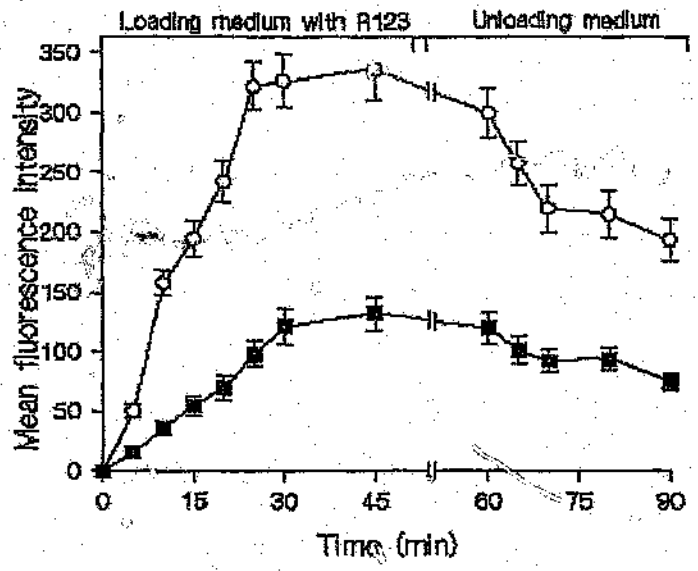


Figure 8.7 : Time course of rhodamine 123 uptake and efflux by *P.falciparum* (FCR-3) infected (O)and uninfected (■) red blood cells. Fluorescence is a measure of quantitiy of cell-associated dye. Each point represents the mean fluorescence intensity in one experiment.

Both the chl quine-resistant (FCR-3) and chloroquine-sensitive (3D7a) strains released R123. Chloroquine-resistance appeared to be associated with a more rapid loss of R123. However, the difference between chloroquine-resistant and chloroquine-sensitive strain was small in comparison to RRI values reported for cancer cells (sensitive ~ 1, resistant > 4) (Table 8.1).

Table 8.1: Results of rhodamine 123 release index (RRI) for different strains of *P.falciparum*

Parasite strain	RRI
FCR-3	1.74
3D7a	1.56

The effects of reduced temperature on uptake (fig 8.8.a) and efflux (fig 8.8.b) of rhodamine 123 in the chloroquine-resistant (FCR-3) strain are shown. Both uptake and efflux were greatly reduced at 4°C. The inhibitory effect of low temperature supports the notion of an active transport process rather than simple diffusion out of the cell.

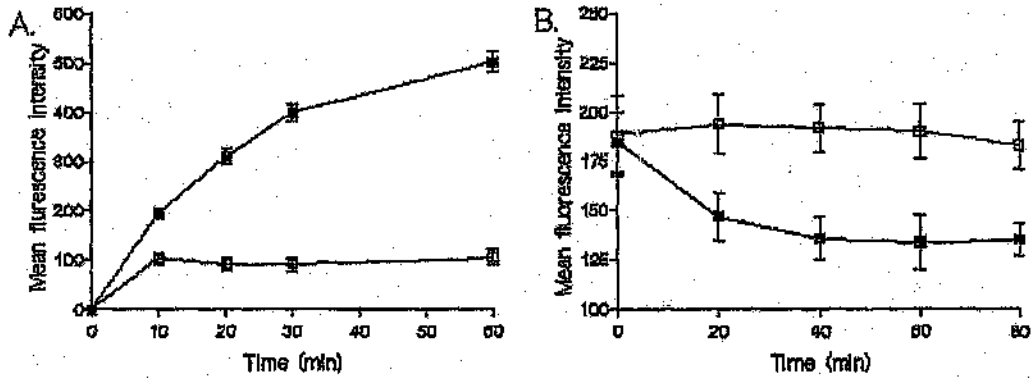


Figure 8.8: Temperature dependence of rhodamine 123 *P.falciparum* A. Uptake and B. Efflux at ■ 37°C and □4°C in chloroquine-resistant. Each point represents the mean fluorescence intensity in one experiment.

Despite differences between R123 release in cancer cells and parasites, R123 release was modulated by classical PgP inhibitors in the parasite. Treatment of parasites with verapamil at concentrations required to modulate drug resistance leads to a marked accumulation of R123 in chloroquine-resistant parasites (fig 8.9a), whereas, accumulation in chloroquine-sensitive parasites is hardly affected by verapamil (fig 8.9b). Treatment with amantadine had no effect on R123 accumulation irrespective of chloroquine sensitivity (fig 8.9).

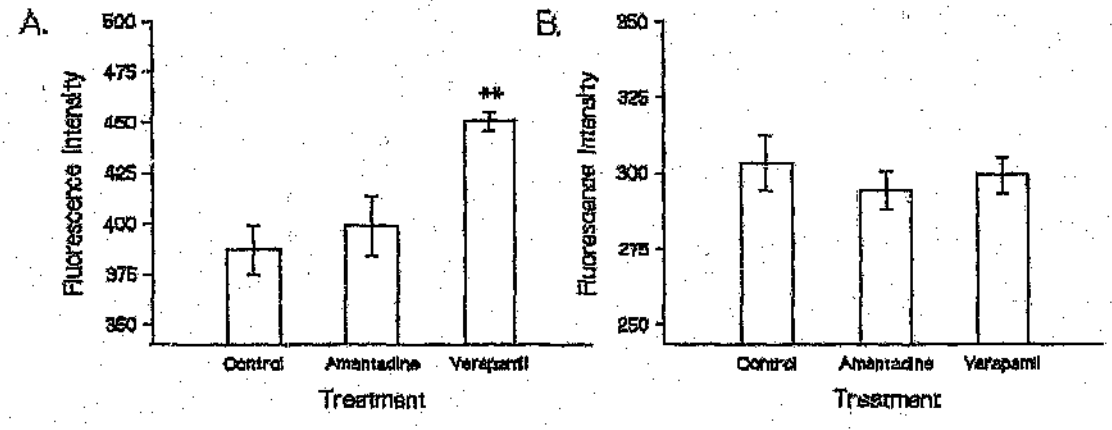


Figure 8.9:R123 release following treatment with amantadine (10 μM) and verapamil (10 μM) in the A. Chloroquine-resistant (FCR-3) and B. Chloroquine-sensitive (3D7a) strain of *P.falciparum* (** $p < 0.001$)

8.4 DISCUSSION

In cancer cell lines, the resistance reversing action of verapamil (and other chemosensitizing drugs) has been fairly well characterised. The chemosensitizing agent is thought to compete with the antitumour drug for a limited number of translocation sites on P-glycoprotein molecule - see figure 8.10) (Ford and Hait, 1993).

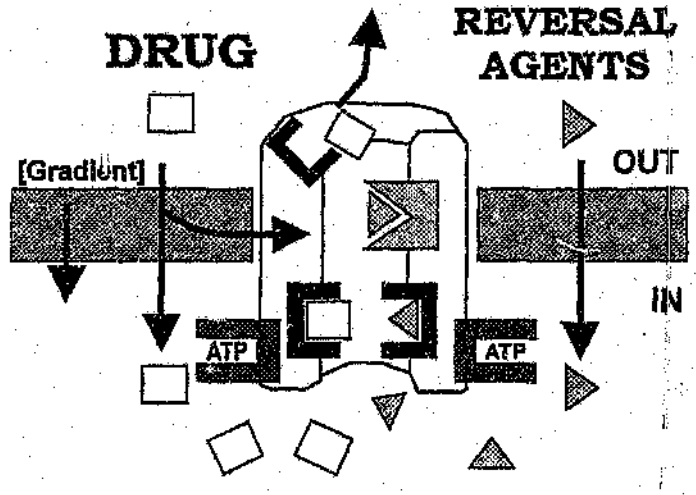


Figure 8.10: Schematic representation of P-glycoprotein

The resistance reversal mechanism in *P.falciparum* is not so well characterised although clearly verapamil and other chemosensitizing agents can cause resistant parasites to accumulate more chloroquine. Initially it was believed that the chemosensitizers inhibited P-glycoprotein in the parasite. In recent years it has become evident that P-glycoprotein represents only a part of a more complex multicomponent system that mediates drug resistance in *P.falciparum*.

P.falciparum contains two genes that are related to the mammalian MDR gene - *pfmdr1* and *pfmdr2*. The *pfmdr2* gene product is expressed on the parasite membrane and expression is unrelated to chloroquine-resistant phenotype (Rubio and Cowman, 1994). The gene *pfmdr1* product (PGH1) is a polypeptide found in all parasite lines irrespective of chloroquine sensitivity. PGH1 is present throughout the asexual life cycle being located at the membrane of the food vacuole of the trophozoite (Cowman, 1991). Initial reports indicated that amplification, overexpression and the presence of specific *pfmdr1* alleles was preferentially associated with some chloroquine-resistant strains of *P.falciparum* (Foote *et al.*, 1989, Foote *et al.*, 1990), however, these results have not been sustained by subsequent findings (Foote *et al.*, 1989, Wellems *et al.*, 1990, Barnes *et al.*, 1992, Peel *et al.*, 1994). The current evidence provides strong support for *pfmdr1* mediating resistance to mefloquine although it is not yet clear how PGH1 could modulate resistance. Various models have been proposed.

Peel *et al.* (1994) theorised that if PGH1 functions as a 'flippase' as suggested for PgP, mefloquine is more tightly associated with membranes than chloroquine and would be expected to function better as a substrate, increasing efflux from the food vacuole, the proposed site of action. However, only minor amounts of PGH1 are localised in the plasma membrane, the mechanism by which the drug would move from the parasite cytoplasm through the parasite plasma membrane, the parasitophorus vacuole membrane and finally through the erythrocyte membrane is unclear. In addition there is no evidence of increase efflux of mefloquine being associated with mefloquine-resistance.

We found the kinetics of rhodamine 123 release in *P.falciparum* is not typical for P-glycoprotein mediated efflux (fig 8.7). Although efflux appears to be an active process (fig 8.8) the difference observed between the resistant and sensitive strains may be attributed to different initial concentration levels rather than increased efflux since resistant parasites exhibit decreased sensitivity to R123 mitochondrial inhibition as determined by IC_{50}s (results not shown). Suggesting the resistance reversal phenomenon is not mediated by P-glycoprotein. Several authors have questioned the presence of the so-called rapid efflux phenotype for chloroquine. Bray *et al.* (1992) reported no rank order correlation between chloroquine-resistance and increased chloroquine efflux. He demonstrated verapamil is effective at decreasing the rate of efflux of chloroquine irrespective of chloroquine sensitivity and that verapamil

accumulation in the presence of chloroquine is increased in both strains suggesting the efflux pump function is identical. He postulated that the capacity of the efflux pump to remove chloroquine from chloroquine-sensitive parasites is swamped by the stronger force for accumulation in these parasites resulting in increased chloroquine accumulation and sensitivity. Both Ginsburg *et al.* (1991) and Ferrari and Cutler (1991) arrived at a similar conclusion based on kinetic models constructed from chloroquine uptake data; i.e. chloroquine-resistance arises from decreased chloroquine accumulation. Ginsburg *et al.* attributed the decreased accumulation to a defective vacuolar proton pump while Ferrari and Cutler speculated there was an accumulating mechanism present in sensitive parasites.

Despite the uncertainty of the importance and relevance of P-glycoprotein in chloroquine-resistance our results confirm P-glycoprotein inhibitors increase chloroquine accumulation (fig 8.5) in resistant parasites. Accumulation of rhodamine 123, a P-glycoprotein substrate, is modulated by P-glycoprotein inhibitors (fig 8.9). R123 may be useful for further studies of the reversal phenomenon, since it is sensitive to modulation by chemosensitizers at concentrations that do not exhibit cytotoxicity, eliminating the influence of cytotoxicity associated with the chloroquine.

Amantadine resistance is associated with PgP, by virtue of the relationship between mefloquine and amantadine sensitivity. However, amantadine has not previously been

identified as a PgP inhibitor and lacks the critical structural requirements identified for PgP inhibition namely two tricyclic rings with an alkyl bridge. However, amantadine is lipophilic and membrane active, features shared by many PgP inhibitors. From the results obtained, amantadine is not functioning in a manner analogous to resistance reversal drugs (PgP inhibitors). Table 8.2 compares the resistance "reversal" properties of amantadine and verapamil.

Table 8.2: Comparison of the resistance "reversal" properties of amantadine and verapamil

	AMANTADINE	VERAPAMIL
Antimalarial activity	5-300 μM	6-21 μM
Resistance ratio (CQ-S/CQ-R)	59.4	3.5
+ chloroquine (CQ-R)	synergistic	synergistic
+ chloroquine (CQ-S)	synergisitc	additive
Chloroquine uptake (CQ-R)	increased	increased
Chloroquine uptake (CQ-S)	increased	unchanged
R123 accumulation (CQ-R)	unchanged	increased
R123 accumulation (CQ-S)	unchanged	unchanged

8.5 SUMMARY

The intrinsic antimalarial activity of amantadine is distinct from the intrinsic activity of the chemosensitizers. The synergistic reaction of amantadine with chloroquine differs from the resistance reversal associated with the chemosensitizers.

CHAPTER NINE - DISCUSSION

9.1 INVERSE RESISTANCE

Amantadine exhibits anti-plasmodial activity, this activity is associated with the chloroquine-resistant phenotype in *P.falciparum in vitro* (IC_{50} = 5 μM). The concentrations exhibiting anti-malarial activity against the chloroquine-resistant strains of *P.falciparum in vitro* correlates with plasma concentrations attainable in man following a normal dosage regimen (0.6 - 7.3 μM) (Greenblatt *et al.*, 1977). An inverse relationship between chloroquine sensitivity and sensitivity to a number of agents has previously been noted e.g. mefloquine (Barnes *et al.*, 1992) and reversal agents (Ryall, 1987), however, the magnitude of the effect (resistance ratio) has been small (resistance ratio of mefloquine = 0.4) in comparison to the effect seen in amantadine (resistance ratio = 0.02). The relationship between amantadine and chloroquine sensitivity appears unique. We have coined the term "inverse resistance" to describe this new category of drugs (Evans and Havlik, 1994).

The identification of "inverse resistance" agents may offer an alternative strategy for the treatment and prophylaxis of chloroquine-resistant malaria. An "inverse resistance drug"-chloroquine combination would be expected to provide a two prong attack against the malaria parasite; the inverse resistance drug providing protection against chloroquine-resistant isolates while chloroquine provides protection against all other isolates. Based on the *in vitro* combination studies of amantadine and chloroquine (chapter 5), such a combination is not expected to be antagonistic. However, comprehensive toxicity testing

to determine any adverse drug interactions between the agents would be required before such a combination could be considered clinically.

In addition to a new clinical approach, the characterization of the inverse resistance mechanism of amantadine may provide a clearer understanding of the mechanism of action of chloroquine as well as how chloroquine-resistance arises and thus facilitate the development of alternative strategies for combating resistance.

The inverse resistance relationship between amantadine and chloroquine is difficult to reconcile mechanistically but probably is connected to the mechanism(s) mediating chloroquine-resistance. The only consistent feature which distinguishes sensitive from resistant parasites is the level of chloroquine accumulation within the trophozoite (Geary *et al.*, 1986). To understand the mechanism(s) involved in the inverse resistance phenomenon it is necessary to first define the mechanism(s) of action of amantadine against the chloroquine-resistant strains. A number of strategies were employed based on the previously described properties of amantadine as well as proposed targets of chloroquine.

9.2 MORPHOLOGICAL EFFECTS

Based on the early lysosomal changes i.e. swelling and vesiculation observed following amantadine treatment, it is clear that amantadine accumulates in the food vacuole of the parasite irrespective of chloroquine sensitivity (fig 5.6). The accumulation of amantadine

in the food vacuole is expected due to the weak base nature of the drug (De Duve *et al.*, 1974) . Briefly, the membrane permeable (neurtral) weak base distributes evenly throughout the cell. On entering an acidic compartment (food vacuole) it is immediately protonated and therefore converted into a membrane impermeant (charged) form. The continuing diffusion gradient for the neutral species drives the selective concentration of the charged weak base in the acidic organelle. Amantadine is expected to accumulate to millimolar levels in the food vacuole at physiological concentrations. The food vacuole of the parasite is the "metabolic headquarters" of the parasite carrying out a variety of specialized functions - see figure 1 (Olliaro and Goldberg, 1995), therefore high concentrations of drug are anticipated to exert toxic effects on the normal functioning of this organelle.

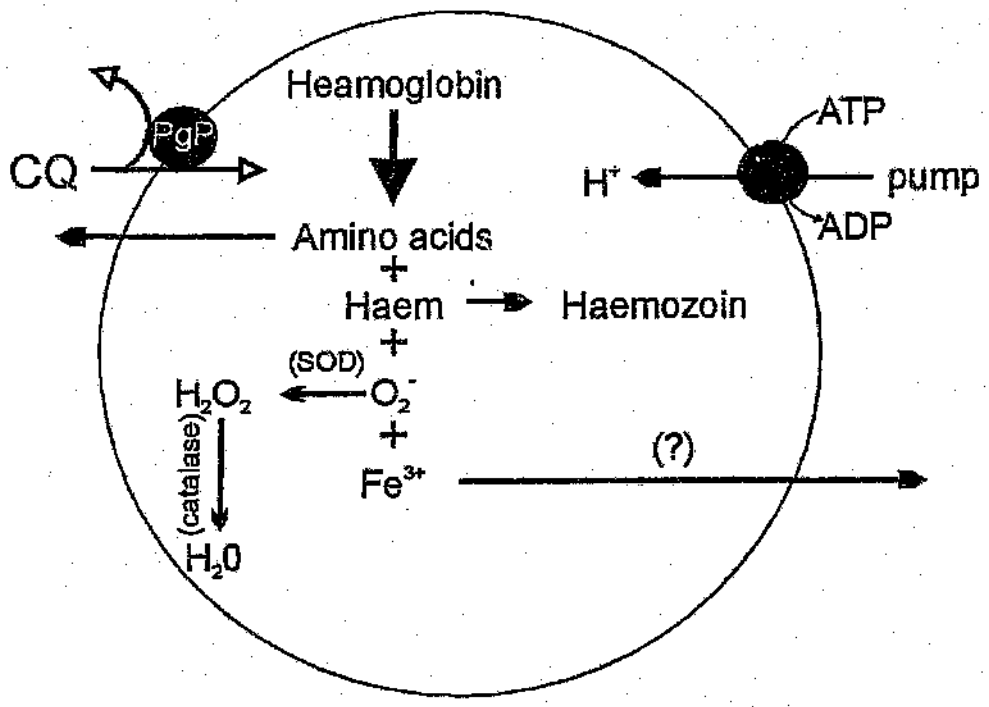


Figure 9.1: The *Plasmodium* digestive food vacuole

9.3 THE EFFECT OF AMANTADINE ON FOOD VACUOLE FUNCTION

9.3.1 HAEMOGLOBIN DIGESTION

There is no morphological evidence that amantadine interferes with haemoglobin digestion, the primary function of the food vacuole. Morphological observations show that the vacuolar changes are not associated with an accumulation of undigested haemoglobin or absence of haemozoin. This is in contrast with the quinolines, chloroquine treatment is associated with accumulation of undigested haemoglobin and absence of pigment (Rosenthal, 1995). Inhibition of trophozoite haemoglobin degradation is believed to be the primary mechanism of action of chloroquine resulting in the parasite starving and initiating other metabolic disturbances (Zarchin *et al.*, 1986).

This finding is supported by the observation that the combination of amantadine with leupeptin, a cysteine protease inhibitor, which has been demonstrated to block parasite development by inhibiting haemoglobin digestion (Rosenthal *et al.*, 1991), is additive (fig 4.9).

9.3.2 ATPase PUMP

Accumulation of lysosomotropic agents in the lysosomes of mammalian cells results in alkalinization of these organelles leading to cell death (Poole and Ohkuma, 1981). The acidic pH of the parasite food vacuole is maintained by a dynamic equilibrium between proton leakage and a vacuolar ATP-dependent proton pump (Ginsburg and Stein, 1991).

Amantadine treatment is expected to produce alkalinzation of the food vacuole. Treatment with amantadine resulted in an increase in the vacuolar pH (fig 6.10). However, the effect of amantadine was transient (fig 6.9) and insufficient to inhibit the functioning of the parasite vacuolar enzymes (table 6.1), which have an acidic optimal pH (Slater, 1[illegible]). There is no evidence that amantadine interferes with the functioning of the proton pump, which has been implicated in chloroquine-resistance. This effect contrasts with the permanent effect observed with the quinolines (fig 6.9). However, the alkalinizing effect of quinolines is also insufficient to account for the antiparasitic effect (table 6.1). The permanent effect observed supports the evidence that the quinolines interfere with the functioning of the ATPase proton pump (Choi and Mego, 1988).

9.4 AMANTADINE ACCUMULATION VIA pH GRADIENT

The accumulation of amantadine in the food vacuole was explored further by investigating the effect of inoculum size and extracellular pH on the amantadine sensitivity and comparing these results to the effects of these parameters on chloroquine sensitivity in chloroquine-resistant and chloroquine-sensitive strains of *P.falciparum*.

9.4.1 INOCULUM EFFECTS

Accumulation of amantadine in the food vacuole, via the pH gradient, is expected to deplete the concentration of extracellular drug. In order to compensate for this depletion, the initial drug concentration required to kill 50 % of the parasites (IC_{50}) should increase as inoculum increases in order to attain lethal intracellular levels (Geary *et al.*, 1990).

Inoculum effects conforming to this model, were obtained for chloroquine irrespective of chloroquine sensitivity (fig 3.8) and amantadine against chloroquine-sensitive strains (fig 3.7). However, inoculum effects of amantadine in the chloroquine-resistant strain (fig 3.7) suggested that factors in addition to food vacuole concentration may be involved in activity against these strains.

9.4.2 pH EFFECTS

The driving force for the accumulation of amantadine in the food vacuole is the pH gradient, therefore changes in the extracellular pH will result in changes in drug accumulation (Geary *et al.*, 1990). The susceptibility to chloroquine, irrespective of chloroquine sensitivity (fig 6.3) and amantadine in the the chloroquine-sensitive strain (fig 6.4b) is linearly dependent on the extracellular pH, as predicted by the pH gradient model, however, amantadine sensitivity in the chloroquine-resistant strain is non-linearly dependent. This unusual relationship between amantadine sensitivity and extracellular pH again suggests factors in addition to the concentration of amantadine in the parasite food vacuole are contributing to the antimalarial activity against chloroquine-resistant strains.

9.5 MEMBRANE ASSOCIATED AMANTADINE

Despite accumulation of amantadine in the food vacuole, several lines of investigation suggested that the antimalarial activity of amantadine may be independent of the concentration of amantadine in the food vacuole, particularly against the chloroquine-

resistant strains. This finding was confirmed by the observation that the membrane-associated component of amantadine is critical for antimalarial activity (fig 7.8).

Amantadine intercalation into the membrane is expected to result in interactions with both the phospholipid fatty acyl chains and electrostatic binding with the polar head groups resulting in the physical properties of the membrane being altered. These changes may produce pertubations in the conformation of integral proteins and/or the energetics associated with their functional activity (e.g. ion conductance and ligand binding) (Mason, 1993).

The ability of amantadine to partition into a membrane is expected to be dependent on its shape and charge as well as on the compostion of the membrane (Mason, 1993). Structure activity studies confirm that the shape and size of the hydrocarbon cage and the hydrophilic group are exceedingly important for the antimalarial activity of aman..dine (table 3.3, fig 6.7). Chloroquine-resistance has been shown to be associated with changes in membrane composition (the biochemical basis for this is unknown) (Shalmiev and Ginsburg, 1993). Changes in the ability of amantadine to partition into the parasite membrane and influence the functioning of a as yet unidentified protein may account for the altered sensitivity to amantadine between different strains. The unusual relationship between amantadine sensitivity and extracellular pH may be explained by alterations in the ability of amantadine to partition into the membrane at different pHs due not only to

changes in the charge of amantadine *per se* but also changes in the charge distribution of the membrane itself.

9.6 MEMBRANE EFFECTS OF AMANTADINE

Amantadine is membrane active compound and expected to intercalate with the membrane producing changes in membrane functioning. However, there is no evidence that amantadine inceases fluidity of the erythrocyte membrane (fig 7.1) the effect of amantadine on other membranes is unknown. Although, amantadine does not inhibit the nutrient permeation pathways of the parasite directly (fig 7.6), one of the first biochemical changes apparent in the parasite following amantadine treatment is an interference with nutrient uptake (fig 7.7).

Nutrient uptake is due to the parasite extensively modifying the environment beyond its own plasma membrane by exporting proteins and lipids to the host via plasmodial transport pathways. The transport pathways have not been well characterized. Labeling of *P.falciparum* infected erythrocytes with flouorescent probes has revealed a complex network of membranes in the erythrocyte cytoplasm termed the tubulovesicular membranes (TVM) through which proteins move to the surface of the infected erythrocyte (Elmendorf and Haldar, 1994) - see figure 2.

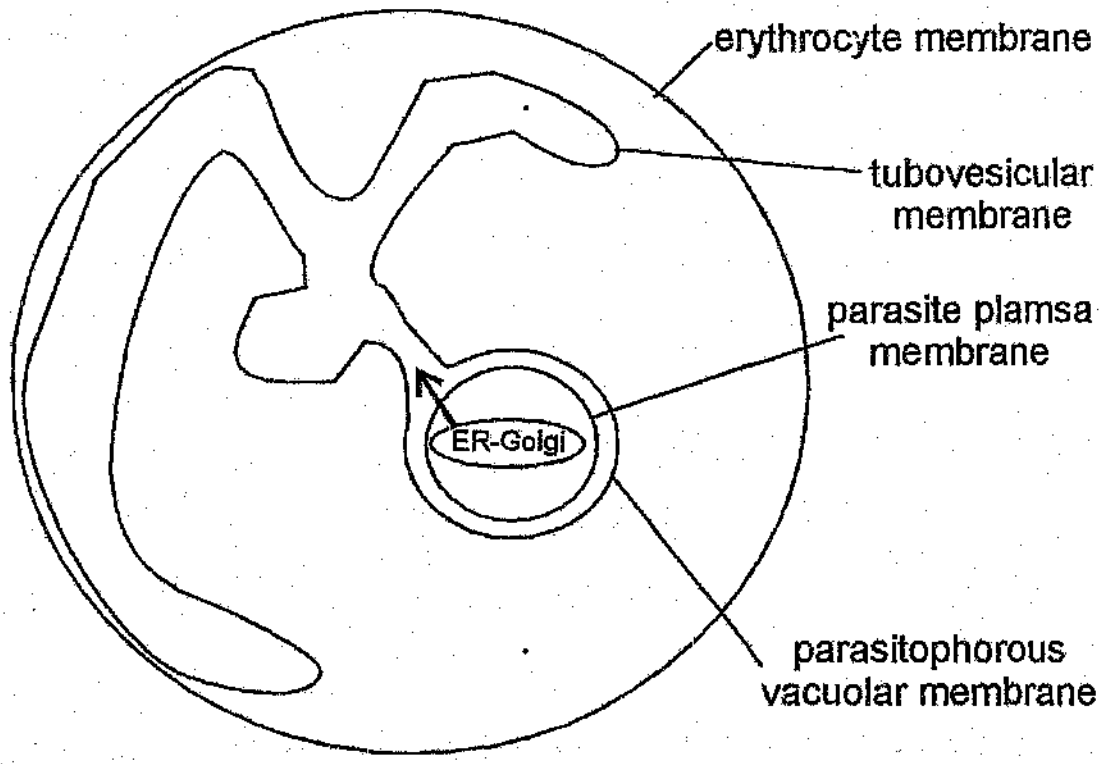


Figure 9.2 : Schematic representation showing secretory protein export from the parasite

The kinetics of protein secretion to the plasma membrane are relatively rapid (Benting *et al.*, 1994). Protein export form the parasite is observed during all stages of asexual parasite development (Benting *et al.*, 1994) which corresponds to the stage specificity of amantadine (chapter 6). Early rings can perform all metabolic functions necessary for their immediate survival in the absence of continued secretory export, but cannot sustain or alter cell functions in a manner necessary for maturation. Blockage of protein export in the trophozoite results in parasite death.

Although the evidence is circumstantial, protein secretion may be the target of amantadine, since stage and time dependent effect of amantadine (chapter 6) correlate

well with those predicted if the parasite protein secretion is blocked. The interaction with brefeldin A, a protein secretion blocker, is antagonistic (fig 4.8). Differences in response to strains showing different chloroquine sensitivities may be due to the absence or presence of a protein involved in chloroquine transport.

9.7 RESISTANCE REVERSAL

Amantadine displays several features associated with agents which "reverse" chloroquine resistance in *P.falciparum*. Although amantadine has not been identified as a P-glycoprotein inhibitor (a characteristic of this diverse group of compounds) these similarities and the association of amantadine resistance with P-glycoprotein, propted us to compare the characteristics of amantadine and verapamil with regards to inherent anti-plasmodial activity and synergy with chloroquine. The IC_{50} values of amantadine and verapamil against the chloroquine-resistant strain (FCR-3) were similar (fig 8.1), however, although verapamil also showed decreased effect against chloroquine-sensitive strains the difference was small (fig 8.1). Combinations of amantadine or verapamil with chloroquine resulted in a synergistic drug interaction in the chloroquine-resistant strain (fig 8.5 and fig 8.6), but only amantadine showed synergy in the chloroquine-sensitive strain (fig 8.6), verapamil's effect was additive (fig 8.5). Both drugs increased uptake of radiolabelled chloroquine in the chloroquine-resistant strain (fig 8.5 and fig 8.6) but only amantadine increased chloroquine uptake in the chloroquine-sensitive strain, the concentrations which increased chloroquine uptake weres shifted to the right (fig 8.6), paralleling the difference in drug sensitivity. An amantadine-verapamil combination

produced an additive response (fig 8.2). The principle mechanisms postulated for "reversal" are a block of the drug efflux pump, P-glycoprotein and/or alkalinization of the food vacuole. Using rhodamine 123 as a probe for P-glycoprotein activity, we found verapamil was able to inhibit rhodamine efflux in the micromolar range in the chloroquine-resistant strain but amantadine had no effect on rhodamine efflux in either strain at concentrations as high as 1 mM (fig 8.9). Acridine orange was used to probe pH changes in the food vacuole. Alkalinization of the vacuole by verapamil was observed in the micromolar concentration range compared with a millimolar range seen with amantadine. Both the intrinsic antimalarial activity and the mechanism of resistance "reversal" of amantadine appears to be unique.

9.8 CONCLUSION

Amantadine exhibits antimalarial activity against *P.falciparum* at concentrations attainable in humans following a normal dosage regimen. The antimalarial activity is associated with chloroquine-resistance due to this unique relationship it has been classified in a new category of antimalarial agents termed "inverse resistance" drugs. The mechanism of action of amantadine remains unclear - however, it clearly involves a membrane interaction.

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